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THE CELLULAR METABOLISM AND LETHAL EFFECTS
OF 1- β -D-ARABINOFURANOSYLCYTOSINE IN THE
PRESENCE OR ABSENCE OF OTHER CYTOTOXIC COMPOUNDS

by



GILLES J. LAUZON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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FALL, 1979

THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE CELLULAR METABOLISM AND LETHAL EFFECTS OF 1- β -D-ARABINOFURANOSYLCYTOSINE IN THE PRESENCE OR ABSENCE OF OTHER CYTOTOXIC COMPOUNDS submitted by Gilles J. Lauzon in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

TO PATRICIA AND NICOLAS

ABSTRACT

The metabolism and antiproliferative effects of the antileukemic agent 1- β -D-arabinofuransolycytosine (araC) were studied in a variety of cell types in the presence or absence of 3-deazauridine (DU), hydroxyurea (HU), pyrazofurin (PF) and high concentrations of deoxythymidine (dThd). In all cell types studied, the conversion of araC to araC-5'-diphosphate choline (araCDPcholine) and araC-5'-diphosphate ethanolamine was evident. AraCDPcholine was characterized by (1) chromatographic behavior, (2) chemical and enzymatic hydrolysis, (3) phosphorus content and (4) incorporation of labelled precursors. In accordance with the established route for the synthesis of cytidine-5'-diphosphate choline by phosphorylcholine cytidylyltransferase [EC 2.7.7.15], it was found that the formation of araC-5'-triphosphate (araCTP) preceded that of araCDPcholine. In all cell types studied, araCTP and araCDPcholine were the major cellular araC anabolites.

RPMI 6410 cells cultured in the presence of 9 μ M DU (1) were "sensitized" to the lethal effects of araC, (2) were depleted of cellular cytidine phosphate concentrations within 6 hr, (3) took up several-times more araC in short term assays than did untreated cells and (4) became synchronized at a locus in S phase. When cells were cultured with araC or araC and DU together, it was observed that (1) DU enhanced the cellular formation of araC phosphates and (2) cell kill in the presence of araC and DU together was 10-fold greater than the sum of the lethal effects resulting

from exposure to araC or DU separately. The biochemical basis of the DU-dependent stimulation of araC anabolism appeared to involve, at least in part, enhancement (2- to 3-fold) of deoxycytidine kinase activity in unfractionated extracts of DU-treated cells. It was proposed that the sensitization of RPMI 6410 cells to araC lethality following exposure to DU probably derived from (1) stimulation of araC phosphate formation and (2) accumulation of cells at a locus in S phase, the cell cycle stage sensitive to araC lethality.

HU and high concentrations of dThd likewise enhanced araC anabolism in many cultured cell types. The dCyd kinase activity present in extracts of HU-treated (4 hr or longer) RPMI 6410 cells was greater than that in extracts of untreated cells. PF did not enhance significantly araC anabolism but increased the proportion of araC-derived radioactivity present in the form of araCDP choline.

When RPMI 6410 or HeLa cells cultured with araC were transferred to drug-free medium, araC anabolites were broken down to araC which then appeared in the culture medium; the time course of the decay of araC metabolites from these cells was biphasic. Nitrobenzylthioinosine, an inhibitor of nucleoside transport, did not affect significantly araC catabolism nor the outward flux of araC. However, dipyridamole effectively inhibited the latter processes.

When attempts were made to apply these findings to the treatment of leukemic mice, the following were observed: (1) a profound host toxicity was shown when DU was administered prior to araC, and low drug concentrations which circum-

vented this toxicity showed no interaction between araC and DU with respect to toxicity toward leukemic cells, and (2) combinations with HU and araC were sequence-dependent: when HU was given 3 or 6 hr prior to araC or 6 hr after araC, therapeutic effects were greater than the sum of the effects of araC and HU administered alone. Application of the inhibitory influences of dipyridamole on araC catabolism to the therapy of murine neoplasms remains to be explored.

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LIST OF ABBREVIATIONS

araC: 1- β -D-arabinofuranosylcytosine

araCMP, araCDP, araCTP: 5'-mono-, di- and triphosphate esters
of araC

araCDPcholine: 1- β -D-arabinofuranosylcytosine 5'-diphosphate
choline

araCDPethanolamine: 1- β -D-arabinofuranosylcytosine 5'-diphos-
phate ethanolamine

CMP, CDP, CTP: 5'-mono-, di- and triphosphate esters of
cytidine

Cyd: cytidine

CH: cycloheximide

dCMP, dCDP, dCTP: 5'-mono-, di- and triphosphate esters of
deoxycytidine

dCyd: deoxycytidine

DNA: deoxyribonucleic acid

dThd: deoxythymidine

dTMP, dTDP, dTTP: 5'-mono-, di- and triphosphate esters of
deoxythymidine

dUMP, dUDP: 5'-mono- and diphosphate esters of deoxyuridine

dUrd: deoxyuridine

DU: 3-deazauridine

DUMP, DUDP, DUTP: 5'-mono-, di- and triphosphate esters
of DU

EAC: Ehrlich ascites carcinoma

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

L.I.: labelling index

M.I.: mitotic index

NBMPR: nitrobenzylthioinosine

NBTGR: nitrobenzylthioguanosine

OA: orotate

OMP: orotidylate

PEI: polyethyleneimine

PF: pyrazofurin

RNA: ribonucleic acid

Thy: thymine

TLC: thin layer chromatography

I. INTRODUCTION

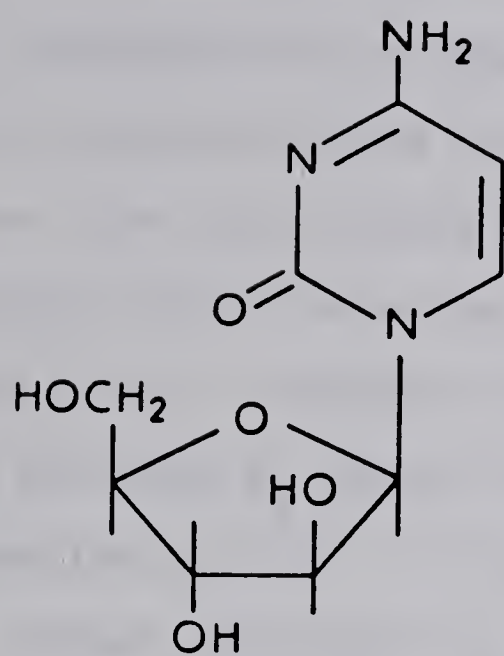
1- β -D-Arabinofuranosylcytosine (araC) has found extensive use in the therapy of human acute leukemia (for review, see (65)). It is the view of the author that continued research directed toward the elucidation of the metabolism of anti-neoplastic agents and the biochemical interactions between drugs can contribute importantly to the development of therapeutic strategies for the use of antineoplastic drugs. In view of the positive contribution of drug combinations with araC to progress in the treatment of human neoplasms (55,65), we have studied the metabolism of araC in the presence and absence of various antineoplastic compounds in an attempt to elucidate biochemical rationales for combination drug treatments involving araC; such drug combinations could then be tested for antineoplastic activity using animal models.

In order to provide a background for the meaningful interpretation of results presented in this thesis, the current understanding of the metabolism and biochemical effects of araC and other relevant cytotoxic compounds is reviewed below.

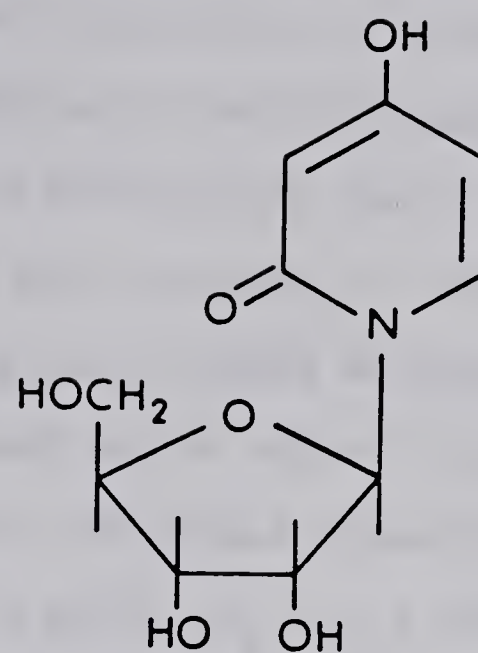
A. AraC

1. Anabolism of AraC.

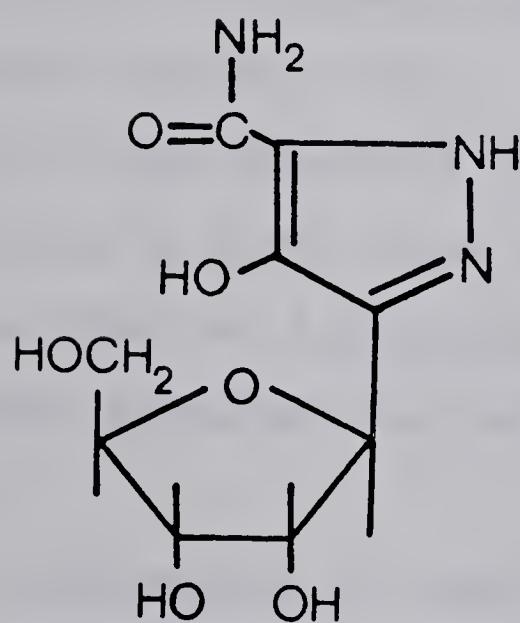
Upon entry into cells, araC (Fig. 1) is converted to the 5'-mono-, di- and triphosphate esters (31, 145) by the sequential actions of deoxycytidine (dCyd) kinase (48,82) deoxycytidylate (dCMP) kinase and nucleoside diphosphokinase



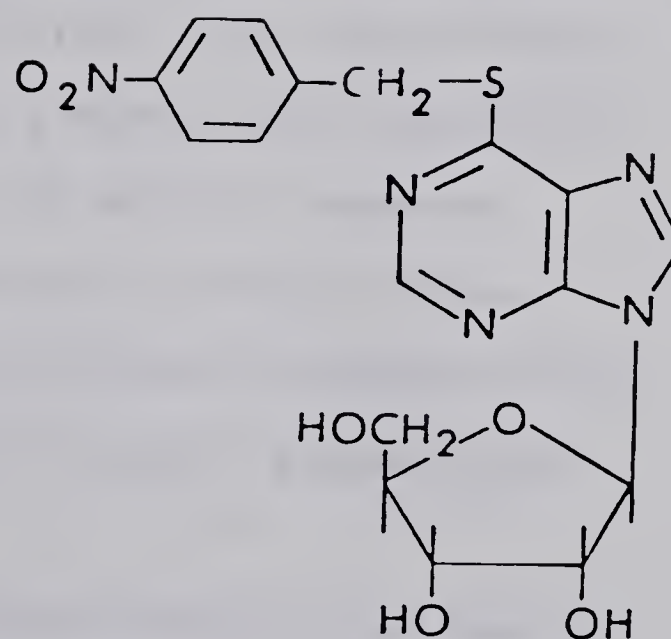
ara C



DU



PF



NBMPR

Figure 1. Chemical Structures

[for review of this topic, see (39)]. In various cell-types and tissues, the 5'-triphosphate of araC (araCTP) has consistently been reported to be the major anabolite.

Deoxycytidine kinase initiates the conversion of araC to its phosphorylated metabolites. Partially purified dCyd kinase from calf thymus displays little specificity for the phosphate donor with ATP, GTP, UTP and dATP serving in this respect (47). Kinetic studies with the calf thymus enzyme have provided K_m values for dCyd and araC of 14 and 40 μM , respectively (103). In the latter study, dCyd was shown to be a potent inhibitor of araC phosphorylation ($K_i = 1.3 \text{ nM}$), whereas araC influenced dCyd phosphorylation only at a much greater concentration ($K_i = 36 \mu\text{M}$).

Deoxycytidine kinase is feedback inhibited by the end product dCyd 5'-triphosphate (dCTP) and, to a lesser extent, by araCTP (28,69,77,103). In this respect, araC phosphorylation is more sensitive than that of dCyd to feedback inhibition by dCTP, 10 μM of the latter causing 49% and greater than 94% inhibition of dCyd and araC phosphorylation by enzyme prepared from murine L5178Y cells, respectively (103).

Incorporation of araC into polynucleotides has been reported by various investigators; however, the amounts detected were very small and stringent identification of the chemical basis of the incorporation was provided in only several instances. Graham and Whitmore (57,58) demonstrated araC incorporation into the DNA of mouse L-cells and found

that 70% of the incorporated nucleoside was released after enzymatic hydrolysis as a 3'-phosphate and, therefore, had been incorporated in 3',5'-phosphodiester linkage in the DNA chain. Evidence for the incorporation of araC into alkali-resistant, internucleotide linkages has also been obtained using L5178Y lymphoma cells (175) and polyoma virus synthesizing systems (67).

These observations were extended to the enzymatic level using DNA polymerases from Walker 256 carcinoma (50), mouse fibroblasts (45) and mouse L-cells (58). Evidence was found that araCTP replaced dCTP as a substrate (50) and there was no indication that incorporation of araC brought about chain termination (45,50,58). In contrast, studies by Momparler (101,102) using partly purified mammalian DNA polymerases and Wagar et al. (166) using mammalian nuclei have shown that incorporation of araCTP into DNA terminated polynucleotide chain growth.

2. Catabolism of AraC.

AraC is deaminated to the less cytotoxic (109) product 1- β -D-arabinofuranosyluracil (araU) by the action of cytidine deaminase [cytidine/deoxycytidine aminohydrolase, E.C. 3.5.4.5]; Camiener (22) reported K_m values for araC deamination by enzyme from human liver of 0.12 to 0.16 mM. An effective inhibitor of cytidine deaminase is 3,4,5,6-tetrahydrouridine (51,167); this agent increased the levels of araCTP attained in human leukemic cells exposed to araC in vitro (29,64).

3. Pharmacology of AraC.

Early clinical studies revealed that the urinary excretion product of intravenously administered araC in humans was mainly (about 90%) araU (41); 70 to 80% of the administered dose was recovered in the urine within 24 hr. Further, serum levels of radiolabelled araC were undetectable chromatographically within 5 to 20 min after i.v. administration to patients (41,156); thereafter, all the plasma radioactivity behaved as araU (41). Serum half-lives of araC in the dog, rat, mouse, hamster and human were 95, 43, 37, 37 and 12 min, respectively (110).

When [^3H]araC was injected subcutaneously into normal mice or mice bearing leukemia L1210, radioactivity became distributed within 30 min throughout the body, including the brain (31). Radioactivity in all tissues began to decline within 60 min after araC administration. The human clinical pharmacology of araC administered with other drugs has been reviewed (65).

4. Metabolic Effects of AraC.

i. Inhibition of DNA Synthesis. AraC inhibited the incorporation of deoxythymidine (dThd) into the DNA (Fig. 2) of a variety of mammalian cells in culture (33,35,57,79), while stimulating the conversion of exogenous dThd to dThd phosphates (71). Incorporation of appropriate precursors into RNA and protein was unaffected by araC (57). AraC also inhibited DNA synthesis in the rat embryo in utero (138) and in leukemic leukocytes from patients treated with the nucleoside (40). AraCTP was found to be a com-

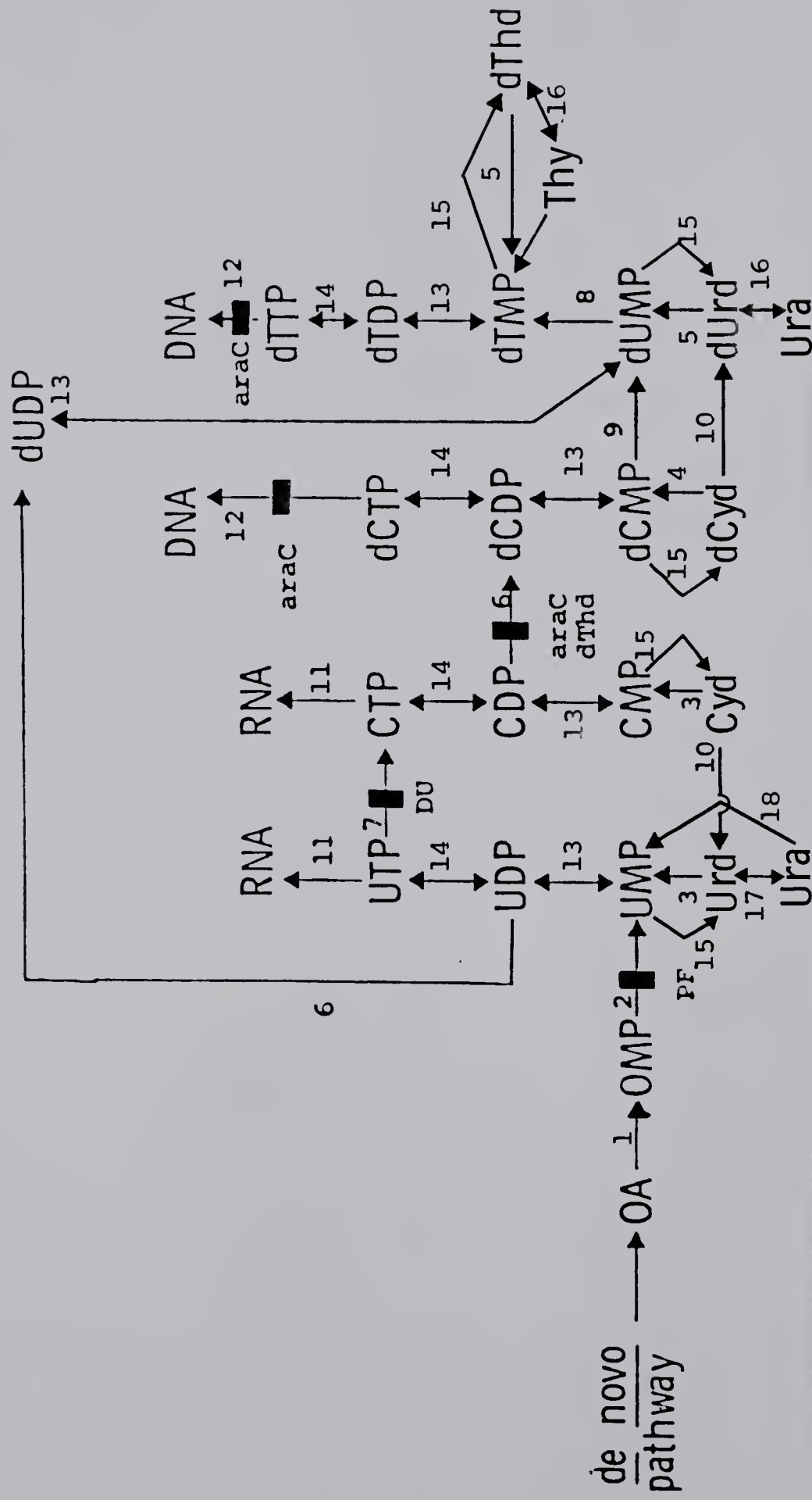


Figure 2. Pyrimidine nucleotide metabolism in animal cells. The figure illustrates (1) interconversions of pyrimidine nucleotides, (2) reactions of salvage pathways leading to pyrimidine nucleotides, and (3) the loci of the inhibitory influences of araC, DU, dThd, and PF. A monograph by Henderson and Paterson (62) provides a review of de novo and salvage pathways of nucleotide metabolism.

Number ^a	Systematic name	Trivial name	Enzyme commission number
1	Orotidine-5'-phosphate pyrophosphate phosphoribosyltransferase	Orotate phosphoribosyltransferase	2.4.2.10
2	Orotidine-5'-phosphate carboxy-lyase	Orotidylate decarboxylase	4.1.1.23
3	ATP:uridine 5'-phosphotransferase	Uridine-cytidine kinase	2.7.1.48
4	NTP:deoxycytidine 5'-phosphotransferase	Deoxycytidine kinase	2.7.1.74
5	ATP:deoxythymidine 5'-phosphotransferase	Deoxythymidine kinase	2.7.1.75
6	2'-Deoxyribonucleoside-diphosphate:oxidized thioredoxin 2'-oxidoreductase	Ribonucleoside diphosphate reductase	1.17.4.1
7	UTP:ammonia ligase (ADP)	CTP synthetase	6.3.4.2
8	-	Deoxythymidylate synthetase	-
9	-	Deoxycytidylate deaminase	-
10	Cytidine aminohydrolase	Cytidine deaminase	3.5.4.5
11	Nucleoside triphosphate: RNA nucleotidyltransferase	RNA polymerase	2.7.7.6
12	Deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase	DNA polymerase	2.7.7.7
13	ATP-CMP phosphotransferase	Cytidylate kinase	2.7.4.14
14	ATP-NDP phosphotransferase	NDP kinase	2.7.4.6
15	5'-Ribonucleotide phosphohydrolase	5'-Nucleotidase	3.1.3.5
16	Deoxythymidine:orthophosphate deoxyribosyltransferase	Deoxythymidine phosphorylase	2.4.2.4
17	Uridine:orthophosphate ribosyltransferase	Uridine phosphorylase	2.4.2.3
18	-	Uracil phosphoribosyl transferase	-

^a numbers correspond to reactions in Fig.2

Key to abbreviations: CMP, CDP, CTP: 5'-mono-, di- and triphosphates of cytidine; Cyd: cytidine; dCMP, dCDP, dCTP: 5'-mono-, di- and triphosphates of deoxycytidine; dCyd: deoxycytidine; DNA: deoxyribonucleic acid; dThd: deoxythymidine; dTMP, dTDP, dTTP: 5'-mono, di- and triphosphates of deoxythymidine; dUMP, dUDP: 5'-mono- and diphosphates of deoxyuridine; dUrd: deoxyuridine; OA: orotate; OMP: orotidylate; RNA: ribonucleic acid; Thy: thymine; Ura: uracil; UMP, UDP, UTP: 5'-mono, di- and triphosphates of uridine; Urd: uridine.

petitive inhibitor of DNA synthesis in nuclei isolated from synchronous S phase HeLa cells (168); the inhibition was completely reversed by dCTP. Evidence was provided that araCTP inhibited DNA chain initiation, elongation and, possibly, ligation.

AraCTP inhibited DNA polymerase partially purified from Ehrlich carcinoma cells (80), human leukemic leukocytes (68), Walker 256 carcinoma cells (50) and mouse L cells (57); in all instances, inhibition was competitive with dCTP. In extracts prepared from murine L-cells, araCTP competed with dCTP in the DNA polymerase reaction, with a K_i of 817 μ M (58).

ii. Inhibition of Ribonucleoside Diphosphate Reductase. The reduction of ADP, GDP, UDP and CDP to the corresponding deoxyribonucleoside diphosphates is catalyzed by ribonucleoside diphosphate reductase (Fig. 2), which plays a central role in the synthesis of DNA precursors (for a review see (137)). The results of Chu and Fischer (33,34) implied that a metabolite of araC inhibited ribonucleoside diphosphate reductase in L5178Y cells in culture: araC reduced the incorporation of Urd and Cyd into DNA and soluble dCyd phosphates while that into RNA and ribonucleotides was unchanged (see Fig. 2). However, subsequent experiments indicated araC causes only slight and transient reductions of cellular dCTP pools in mouse embryo cells (149) and human leukemic leukocytes (68). Inhibition of ribonucleoside diphosphate reductase does not appear to contribute important-

ly to the biological effects of araC in many cell types.

5. Lethality of AraC.

AraC is cytotoxic toward a variety of mammalian cells in culture (33,79,174). Phosphorylated metabolites of araC must be formed intracellularly if araC is to exert its lethal action (34,145). Therapeutic responses of transplantable rodent neoplasms to therapy with araC vary considerably, some being sensitive and others less so (44,169).

The sensitivity of cells to araC is determined by their position in the replication cycle: S phase cells are sensitive and G₁ phase cells are relatively resistant (7,57,73,164,174). Cells exposed to araC during S phase are not "rescued" by provision of dCyd, as are G₁ phase cells (174). Therefore, the proliferative state of a cell population is an important determinant of araC toxicity.

Our present understanding of the relations between (1) the metabolism and metabolic effects of araC, (2) inhibition of cellular proliferation by araC and (3) cell death caused by this nucleoside does not allow satisfactory rationalization of the biochemical events responsible for cell death. As noted above, inhibition of ribonucleoside diphosphate reductase probably does not contribute importantly to the biological effects of araC. Incorporation of araC into DNA does not correlate with the death of cultured murine cells (32,35,36,58); this lack of correlation was established on the basis of concentration and time dependencies of araC incorporation into DNA and araC-induced cell death. On the

other hand, incorporation of araC into light fractions of RNA (2 to 16 S) did correlate with cell death in L5178Y cells (32), possibly as a result of inhibition of histone synthesis (15).

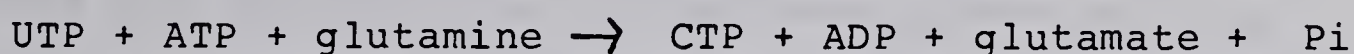
Others have reported a strict correlation between acute cell death by araC and the production of five or more chromatid breaks per metaphase figure (5,71,72). Karon et al. (72) related cell death to the inability to repair chromatid breakage during late S phase, and found no relationship between araC lethality, inhibition of DNA synthesis and unbalanced growth, substantiating earlier results by Graham and Whitmore (57). It is not known whether the reported inhibition by araC of glycoprotein and glycolipid synthesis in hamster embryo cells (61) participates in araC cytotoxicity. Relationships between the biochemical effects of araC and cytotoxicity have been reviewed by Cohen (37,38).

B. 3-Deazauridine

3-Deazauridine (DU, Fig. 1) inhibits the proliferation of microbial cells and various mammalian cell lines (70,97,139). As a therapeutic agent, DU increased the survival time of mice bearing mouse leukemia L1210 (10,12,19). DU also possesses antiviral activity (147). The cellular metabolism of DU involves phosphorylation to the 5'-mono-, di- and triphosphates, as demonstrated in broken cell preparations and intact cells of mouse leukemia L1210 and the Ehrlich ascites carcinoma (EAC) (165). The glycosidic bond

of DU was not cleaved in crude extracts of EAC and microbial cells. DU was not incorporated into DNA or RNA in EAC or leukemia L1210 cells and formation of the 2'-deoxy derivatives of DU in these cell was not in evidence (165). Results from this laboratory using RPMI 6410 cells have shown that resistance to DU was associated with deficiency in uridine (Urd) kinase (97), indicating that (1) DU is a substrate for Urd kinase and (2) phosphorylation is essential for the expression of DU toxicity.

Cytidine 5'-triphosphate (CTP) synthetase catalyzes the conversion of UTP to CTP (Fig. 2) in a reaction requiring ATP, L-glutamine, Mg^{2+} and a sulfhydryl compound (141):



DUTP, the 5'-triphosphate of DU, is a potent inhibitor of CTP synthetase prepared from calf liver and leukemia L1210 cells (19,93). The inhibition is competitive with respect to UTP and a K_i of 5.3 μM was obtained for the DUTP inhibition of the reaction catalyzed by the calf liver enzyme (93). The following observations indicate that growth inhibition by DU results from interference with CTP synthetase (see Fig. 2): (a) growth inhibitory effects of DU toward microbial cells (139) and mammalian cells in culture (19,93) were partially reversed by Cyd, uridine and dCyd, but not by deoxyuridine (dUrd) or dThd, and (b) pronounced depletion of cellular Cyd and dCyd phosphates resulted when leukemia L1210 cells were cultured in the presence of DU (19). The latter workers have also provided evidence

for the inhibition of ribonucleoside diphosphate reductase by DU 5'-diphosphate.

The consequences of the inhibition of CTP synthetase are numerous, as various reports have shown. The depletion of cellular Cyd and dCyd phosphates was associated with inhibition of RNA and DNA synthesis, but not of protein synthesis in leukemia L1210 cells (19). Also affected were cellular concentrations of CDPcholine and CDPethanolamine (19); CTP depletion may well interfere with phospholipid biosynthesis, a possibility which remains to be investigated. Cytidine-3',5'-monophosphate (cyclic CMP) has been isolated from extracts of leukemia L1210 cells (9) and was shown to initiate the proliferation of stationary phase cells in vitro (13). Since the enzymatic synthesis of cyclic CMP from CTP in mammalian tissues remains to be demonstrated conclusively (52), it is not known whether (1) the depletion of CTP pools influences the cellular levels of cyclic CMP and (2) alteration of cyclic CMP concentrations contributes to DU cytotoxicity.

In mice treated with toxic doses of DU, decreased mitotic activity in bone marrow and spleen was evident, as was severe injury to the epithelium of the small intestine. (12). The toxicity of DU was greater in female mice than in males and was reduced in females treated with testosterone (11,12,14); it was suggested that an action of testosterone alleviated DU toxicity toward the mouse intestinal epithelium (12).

DU is presently undergoing Phase I clinical evaluation; an earlier study investigated the disposition of DU in mice: greater than 50% of DU administered was recovered as unchanged drug in the urine within 2 hr (42). DU-associated radioactivity was distributed to all tissues, but liver, kidney and small intestine showed the highest concentrations; 5'-mono, di and tri phosphate esters of DU were formed in all tissues, and alteration of neither the pyridine nucleus nor the ribose moiety was evident in the liver and kidney.

C. Hydroxyurea

The inhibition by HU of the proliferation of mammalian cells in culture was partially reversed by dThd, dUrd and dCyd, but not by Cyd or Urd (100), suggesting interference with the synthesis of deoxyribonucleotides. In cultured cells, HU caused inhibition of DNA synthesis, but not of RNA or protein synthesis (2,146,170,171). Young et al. (173) and Adams and Lindsay (2) demonstrated that dAdo, dGuo and dCyd, but not Ado or Guo partially reversed the inhibition of DNA synthesis in cultured cells.

In accordance with the above-mentioned experiments in which deoxyribonucleotides were shown to reverse growth inhibition caused by HU, the latter was shown to inhibit the activity of ribonucleoside diphosphate reductase (104, 161) probably as a result of chelation of iron in the B₂ subunit of the enzyme (104,172). A recent report showed that the provision of the four deoxyribonucleoside 5'-triphosphates

reversed HU inhibition of DNA synthesis in Chinese hamster ovary cells rendered permeable by treatment with lysolecithin (96), suggesting that HU affected neither DNA synthesis per se nor DNA structure in a manner which interfered with DNA replication. HU has been shown to inhibit glycoprotein and glycolipid biosynthesis in hamster embryo cells (61); the relationship of these effects to HU cytotoxicity is not known.

Culture in the presence of HU reduced the concentrations of dGTP and dATP in mouse embryo cells while those of dCTP and dTTP were increased (149). In synchronous Chinese hamster cells, the presence of HU failed to inhibit the S phase-specific enhancement of dTTP, dCTP and dGTP concentrations, but completely inhibited the corresponding synthesis of dATP (1963). Likewise, the presence of HU reduced cellular concentrations of dATP in PHA-stimulated lymphocytes in culture; levels of dTTP increased while those of dGTP and dCTP remained unchanged (157). Collectively, these results imply that HU inhibition of ribonucleoside diphosphate reductase is selective for the reduction of ADP and possibly GDP; no evidence exists for inhibition by HU of the reduction of CDP and UDP in whole cells.

The lethal effect of HU, like that of araC, is cell cycle dependent: only those cells in S phase are lethally damaged by HU (148). Cells in G_1 and G_2 states survive HU exposure and progress to the G_1/S border, where they accumulate. Hence, HU is a useful cell cycle synchronizing

agent which causes cells to accumulate at a locus in the cell cycle just prior to initiation of DNA synthesis (2,120, 136,148).

D. Pyrazofurin

Pyrazofurin (PF, Fig. 1), an antibiotic produced by Streptomyces candidus (114), is active against several solid tumors (155) and the growth of a variety of cultured cells is inhibited by PF (20,131). After conversion to the 5'-monophosphate by adenosine kinase, PF is further metabolized to the 5'-di and triphosphate derivatives (20,21). The incorporation of PF-associated radioactivity into nucleic acids of cultured L5178Y cells was minor and proof that such activity was present in the form of PF was not obtained (20). Inhibition of orotidylate decarboxylase by PF 5'-monophosphate [Fig. 2] (20,114) is the basis of the accumulation of orotate and orotidylate in PF-treated cells. Reduction in cellular concentrations of CTP and UTP in PF-treated cells (20) is the evident basis for inhibition of nucleic acid synthesis and of protein synthesis (131). Consistent with this mechanism of PF-action is the reversal of PF growth inhibitory effects by Urd (20,131). The clinical pharmacology of PF has been studied by Ohnuma et al. (114) and Cadman et al. (29). Blockade of orotate metabolism in patients was greater than 99% 24 hr after a single i.v. dose of PF (200 mg/sq m), suggesting very tight binding of PF 5'-monophosphate to orotidylate decarboxylase (20).

E. Inhibition of Cellular Replication by Deoxythymidine.

Deoxythymidine in high concentrations (0.1 mM and higher) is inhibitory to animal cells in culture (59,91,92, 107,108); phosphorylated derivatives of dThd give rise to the inhibition (107). Inhibition of cellular replication in L5718Y cell cultures by dThd was reversed by dCyd, but not by Cyd or other nucleosides (107). Concentrations of dCTP were reduced in dThd-treated cells, whereas levels of dTTP, dATP and dGTP were increased (1,8,91,94). Thus, reduced concentrations of dCTP would appear to explain the inhibition of DNA synthesis by dThd in mammalian cells in culture (8,91,95). In cell extracts, dTTP was shown to inhibit the reduction of CDP to dCDP by ribonucleoside diphosphate reductase (105), without interfering with the reduction of ADP or GDP.

F. Inhibition of Nucleoside Transport

1. General.

In this presentation, "transport" will refer to the mediated passage of nucleosides across the plasma membrane and "uptake" will include both transport and intracellular metabolism of nucleoside permeants. Transport of Urd and dThd in the absence of intracellular metabolism was demonstrated with human erythrocytes (25,117). The transport process exhibited the characteristics of facilitated diffusion, including (1) saturability of rate with respect to concentration of permeant, (2) inhibition by compounds structurally

similar to permeant, and (3) demonstration of "trans" effects including counter transport and accelerative exchange diffusion. By means of the latter process, it was shown that the nucleoside transport mechanism of the erythrocyte accepted as substrates both purine and pyrimidine nucleosides. Both araC and DU served as permeants for the erythrocyte nucleoside transporter (25); the transport of araC was also demonstrated in the immature rat uterus (Oliver, 1971). Nucleoside transport and the relationship of this process to nucleoside metabolism have been reviewed by Berlin and Oliver (6).

2. Inhibitors of Nucleoside Transport.

Various thioethers of 6-thioinosine and 6-thioguanosine (127,128) inhibit specifically the permeation of nucleosides; the most studied of these are the S⁶-p-nitrobenzyl derivatives (26,27,121,123,125,126). Nitrobenzylthioinosine (NBMPR, Fig. 1) and nitrobenzylthioguanosine (NBTGR) inhibit the transport of purine and pyrimidine nucleosides in erythrocytes and HeLa cells, but do not affect permeation of nucleobases, sugars or amino acids. The binding of NBMPR to high affinity sites on erythrocyte membranes was evidently responsible for inhibition of nucleoside transport because fractional inhibition of Urd transport was directly proportional to the NBMPR occupancy of these sites (24). Interaction of [³⁵S]NBMPR with high affinity binding sites on HeLa cells also resulted in inhibition of dThd and Urd transport; however, the relationship between site occupancy and inhibition is not simple since a substantial transport

capability remained when total occupancy of the high affinity binding sites was achieved (85). Erythrocytes bind NBMPR with an apparent dissociation constant of 2 nM and to the extent of about 10^4 molecules/cell (24); corresponding parameters in HeLa cells were 0.15 nM (20°) and 10^5 molecules/cell, respectively (85). The concentration of NBMPR which inhibited uptake of dThd, Ado and Urd by 50% in HeLa cells was approximately 50 nM (27,121,123,125).

Dipyridamole* has been shown to inhibit nucleoside transport in a variety of systems: heart (83), rat hepatoma cells (130) and chick fibroblasts (144). However, the effect is not specific for nucleosides since dipyridamole also inhibits the influx and efflux of phosphate and fucose from mouse leukemia cells (6,78). Thus, it is evident that dipyridamole can alter the activity (inward and outward) of a variety of membrane transport systems.

G. The Cell Cycle

This section describes the fluctuations of certain enzymatic activities and of cellular concentrations of deoxyribonucleotides at different stages of the cell cycle. For detailed reviews of cell cycle events, the reader is referred to monographs by Prescott (135) and Mitchison (99).

*2,6-Bis(diethanolamine)-4,8-dipiperidino-pyrimido-5,4-d-pyrimidine; persantine

1. Variations in Enzymatic Activities During the Cell Cycle.

The activity of some enzymes of deoxyribonucleotide metabolism varies with the stage of the cell life cycle. Among those enzymes are dThd kinase, dCyd kinase and ribonucleoside diphosphate reductase.

i. Deoxythymidine Kinase. In a number of mammalian cell types, dThd kinase activity is lowest during G_1 phase and rises gradually during S phase; activity is maximal (several-fold above G_1 levels) just after the peak of DNA synthesis and declines abruptly at the end of mitosis (4,18,46,54,66,90,118,129,152,159).

The S phase elevation of dThd kinase activity is not the result of stabilization of the enzyme (90), but involves synthesis of new enzyme (18,129). Variation of dThd kinase activity during the cell cycle does not appear to be due to dissociable activators or inhibitors since dThd kinase activities of extracts prepared at different stages of the cell cycle were additive when mixtures were assayed (4,18,118).

ii. Deoxycytidine Kinase. In mammalian cells, the activity of dCyd kinase is lowest during G_1 and increases several fold with progression into S phase to a maximum coincident with the peak of DNA synthesis; this activity declines during G_2 phase and mitosis (18,46,66,129,164). The elevation of dCyd kinase activity following PHA-stimulation of lymphocytes was due to synthesis of enzyme

(129). No new species of dCyd kinase were detectable in mouse parotid glands in which the enhancement of DNA synthesis, dThd and dCyd kinase activities and cellular proliferation occurred synchronously as a result of treatment with isoproterenol (46). The cell cycle variation of dCyd kinase appears not to be a consequence of the presence or absence of dissociable activators or inhibitors (18).

iii. Ribonucleoside Diphosphate Reductase.

During the mammalian cell cycle, ribonucleoside diphosphate reductase activity is lowest in early G_1 , increases several-fold during S phase and reaches a maximum during G_2 (111,162). The presence of actinomycin D during G_1 and early S phases of hamster fibroblasts blocked the increase in activity, indicating that its basis was synthesis of new enzyme molecules (111); the low levels of activity in early G_1 cells were not due to the presence of inhibitory substances (111).

Other cell cycle related enzymes include dCMP deaminase (53), cytosine nucleoside deaminase (164) and DNA polymerase (3). In general, the cellular activities of these enzymes are lowest during G_1 , increase several-fold with progression into S phase to reach a maximum during late S phase and decline thereafter. The activities of deoxyadenosine (dAdo) and deoxyguanosine (dGuo) kinases do not appear to be cell cycle related (18).

2. Deoxyribonucleotides and the Cell Cycle.

Cellular concentrations of deoxyribonucleotides differ with cell cycle stage (16,150,163). Throughout G_1 , cells contain deoxyribonucleoside 5'-triphosphates, but concentrations expand several-fold when cells enter S phase. Cellular concentrations of dATP, dGTP and dTTP are highest after the peak of DNA synthesis, whereas dCTP concentrations are highest during mid-S phase.

H. Objectives of This Study

In the author's view, the following excerpt (from a review by S.S. Cohen (38)), which refers to the agents araC and araA appropriately defines the broad objectives of research in cancer chemotherapy:

"...both basic and clinical investigation are essential to effective development of anticancer agents and [are] the price we must pay if we wish to be effective in chemotherapy. The problem in placing the right drug in the right site at exactly the right time demands a complex interdisciplinary effort..."

A goal of the present study was to enhance the cytotoxicity of araC by combining it with other drugs. To achieve that end, we investigated the anabolism and catabolism of araC in sensitive cells in tissue culture and in mice, and assessed the influence of other drugs upon these processes. The rationale for this approach was that drugs which enhance araC anabolism, or inhibit its catabolism, would be likely candidates for successful combination with this nucleoside for the treatment of neoplastic disease.

A recent report (140) provides evidence that the sensitivity of murine neoplasms to araC is dependent upon the cellular concentrations of araC phosphates achieved and the retention times of these anabolites within the neoplastic cells.

Drug combinations employed in cancer chemotherapy have been chosen mainly on the basis of tolerable toxicities and without particular attention to biochemical rationale. By the use of animal and tissue culture model systems in which drug metabolism can be assessed, researchers can contribute to the development of therapeutically beneficial combinations. Once conditions have been defined at the experimental level, collaboration between basic and clinical researchers can lead to the ultimate objective: to provide utmost therapeutic benefit to the cancer patient.

II. Materials and Methods

A. Chemicals

[5-³H]araC, [5-³H]Cyd, L-[4,5-³H]leucine, [methyl-¹⁴C]choline and [2-¹⁴C]ethan-1-ol-2-amine were purchased from Amersham Corp. (Oakville, Ontario). [5,6-³H]araC, [methyl-³H]dThd, [5-³H]dCyd and [2-¹⁴C]dCyd were obtained from Moravek Biochemicals (City of Industry, Ca.). DU was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Nucleosides, nucleotides, 5'-nucleotidase, apyrase, phosphorylcholine, CDPcholine, CDPethanolamine, ovalbumin, dithioerythritol and p-nitro-phenylthymidine-3'-monophosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). 6-Aminocaproic acid was purchased from the Aldrich Chemical Co. (Milwaukee, Wis.). Cell culture materials were obtained from the Grand Island Biological Co. (Calgary, Alberta). Materials for scintillation counting were purchased from Terochem Laboratories Ltd. (Edmonton, Alberta). Dipyridamole was a gift from Boehringer Ingleheim (Canada) Ltd., (Dorval, Quebec).

B. Maintenance of Cultured Cell Lines

1. RPMI 6410 Cells.

RPMI 6410 cells, a lymphoblastoid B-cell line (98) derived from peripheral blood cells from an acute myelogenous leukemia patient (105), were maintained in static culture in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum. Cell concentrations,

determined with an electronic particle counter (Coulter Electronics Ltd., Hialeah, Fl.), were kept below 5×10^5 cells/ml to assure exponential proliferation; under these conditions, cell numbers doubled every 17 to 19 hr. Every 6 to 8 weeks, cultures were restarted from Mycoplasma-free* stocks stored in liquid nitrogen. Because of the possible presence of nucleosides in undialyzed serum, medium containing 10% dialyzed fetal calf serum was used in all experiments in which RPMI 6410 cells were treated with drugs.

2. HeLa Cells.

HeLa cells were maintained by weekly passage of monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum, 1.8 mM calcium and 2 mM HEPES** buffer (pH 7.4) at 37° in 5% CO₂-air. After 6 to 8 serial passages, cultures were restarted from Mycoplasma-free stocks kept in liquid nitrogen. Spinner cultures, started weekly from trypsinized monolayers, employed calcium-free MEM plus 5% calf serum and 2 mM HEPES buffer (pH 7.4); cell concentrations doubled every 20 to 22 hr when kept below 6×10^5 cells/ml. Replicate monolayer cultures for nucleoside uptake experiments were prepared by innoculating 2-oz prescription bottles (Brockway Glass Co., Brockway, Pa.) with approximately 1×10^6 cells (in MEM plus 10% calf serum, 2 mM HEPES buffer, pH 7.4, and 1.8 mM calcium) from

*Determined by Dr. J. Robertson, Department of Medical Bacteriology, University of Alberta.

**N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

single-cell suspension cultures, obtained by growing cells under continuous agitation with a model El Vibro-Mixer (Chemapec Hoboken, New Jersey). Unless indicated otherwise, cells were allowed to attach during 16-24 hr incubation periods at 37° in 10% CO₂-air after which monolayers contained about 10⁶ cells each.

3. L5178Y Lymphoma Cells.

A cloned line of the L5178Y lymphoma was used (160); cultures were restarted at frequent intervals from frozen stocks and were maintained in static culture in Fischer's medium containing 10% horse serum at 37° in 5% CO₂-air. Cell concentrations doubled every 11 hr when kept below 5 x 10⁵ cells/ml.

4. LS Cells.

LS cells, a line of human, nonleukemic leukocytes obtained through the courtesy of Dr. J.E. Seegmiller (University of California, San Diego, Ca.), were maintained in static culture in RPMI medium 1640 containing 20% fetal calf serum at 37° in 5% CO₂-air. When cell proliferation was exponential, the population-doubling time was about 30 hr.

C. Collection of Peripheral Leukemic Myeloblasts

For the collection of peripheral leukemic blast cells from leukemic patients, fresh blood samples were mixed with 0.25 volumes of Plasmagel (Roger Bellon Laboratories, Neuilly, France) and left undisturbed for 40 min at 21° to allow cell aggregates to settle. Cells were obtained by centrifuging (200 g, 5 min) the unsettled upper portion of the suspension

and were washed once with warmed RPMI medium 1640 containing 10% dialyzed fetal calf serum. Dr. A.W. Belch (Cross Cancer Institute, Edmonton, Alberta) kindly provided blood samples from leukemic patients.

D. Metabolic Studies Using Whole Cells

1. Determination of Acid-Soluble Nucleotides.

For the determination of cellular acid-soluble metabolites, perchloric acid extracts were prepared as follows. Cell suspensions were cooled to 4° and cells were collected by centrifugation (150 x g for 4 min at 4°), washed once and extracted at 4° with 0.4 M perchloric acid (100 μ l/10⁷ cells). After re-extraction of the acid-insoluble residues, the combined extracts were neutralized at 4° with KOH using bromcresol purple as an internal indicator, freed of perchlorate, and weighed to determine volume. Metabolites of labelled compounds in the extracts were determined by chromatographic analysis on MN300 thin layers of polyethyleneimine(PEI)-cellulose (Macherey-Nagel and Co., Darmstadt, West Germany); radioactivity comigrating with appropriate markers was determined as described below. Prior to use, impurities were washed from the PEI-cellulose sheets onto paper wicks by the ascending movement of 4.0 M ammonium formate buffer, pH 7.0; the sheets were subsequently washed in methanol and water to remove ammonium formate.

Table 1 lists the mobilities of various compounds on the PEI-cellulose sheets in the solvent systems used. Solvent

TABLE 1

Relative mobility (R_f)^a on PEI-cellulose
thin layer chromatograms

Compound	R_f in these solvents ^b			
	1	2	3	4
AraCTP	0.04	0	0	0
UTP	0.01	0	0	0
AraCDP	0.23	0	0	0.01
UDP	0.13	0	0	
AraCMP	0.63	0.09	0	0.05
UMP	0.39		0	
AraC	0.91	0.58	0.22	0.82
AraU	0.86		0.58	
Uridine	0.86		0.43	
AraCDPcholine	0.79	0.41	0	0.60
CDPcholine	0.79	0.41	0	0.23
CDPethanolamine	0.77	0.34	0	0.15
P-choline		0.63		

^a Distance migrated relative to solvent front.

^b *Solvent 1:* the following solvents were used sequentially without drying between changes; the front was run to 2 cm above the origin with 1 N acetic acid, then to 8 cm with 0.66 N acetic acid containing 0.33 M LiCl, and finally to 16 cm with 0.66 N acetic acid containing 0.66 M LiCl; *Solvent 2:* 0.1 N acetic acid; *Solvent 3:* ethyl acetate: isopropanol:water (65:22.5:12.5, v/v); *Solvent 4:* 0.1 M ammonium formate containing 2% boric acid (pH 7.4).

system 1 was developed during the present study. Sections of the chromatographic medium were assayed for radioactivity by a combustion-liquid scintillation method; samples were combusted in a Packard Model 306 Sample Oxidizer which traps combustion products in a scintillant mixture. Essentially all of the radioactivity applied to the chromatograms (95% to 105%) was accounted for throughout these experiments.

Ribonucleotides present in the neutralized acid extracts were determined by high pressure liquid chromatography (HPLC) on a 25-cm x 4.5 mm Reeve Angel Partisil-10SAX column (Mandel Scientific, Montreal, Quebec), with the use of a linear gradient of potassium phosphate and potassium chloride (151).*

2. Radioactivity Associated with the Acid-Insoluble Fraction.

To measure the incorporation of [^3H]-araC into polynucleotides, acid-insoluble residues obtained as described above were rinsed 3 times with 4 ml of cold 0.4 M perchloric acid, once with 5 ml of cold 5% (w/v) trichloroacetic acid and dissolved in 0.2 ml of 0.5N KOH; samples of the resulting solutions were spotted on filter paper squares which were assayed for radioactivity by the combustion-liquid scintillation procedure.

*HPLC analyses were performed in the laboratory of Dr. L.W. Brox, to whom we are grateful.

3. Determination of Total Cellular Radioactivity.

Total radioactivity present in cells was determined as follows: washed cell pellets were dissolved in 0.5 N KOH (0.2 ml/10⁷ cells); the resulting solutions were assayed for radioactivity by one of two methods. Method A: the solutions were spotted on paper squares and assayed for radioactivity by the combustion-liquid scintillation procedure. Method B: Solutions were mixed with 13.5 ml of a detergent-xylene fluor* and assayed for radioactivity by liquid scintillation counting. Method A was used when radioactivity associated with leukemic blast cells was assayed since color derived from the small number of erythrocytes present in myeloblast suspensions interfered with direct counting as described in Method B.

E. Enzymatic Characterization of Nucleotide Products

Among the criteria used in the characterization of particular nucleotide products was susceptibility to hydrolysis by snake venom 5'-nucleotidase and potato apyrase. 5'-Nucleotidase reaction mixtures contained, in a total volume of 600 μ l, 500 mM Tris-glycine buffer (pH 9.0), 10 mM MgCl₂, 0.1% mercaptoethanol, 1.5 units of 5'-nucleotidase,

*35 ml ethylene glycol, 140 ml 98% ethanol, 250 ml Triton X-100, 3 g 2,5-diphenyloxazole, 0.2 g 1,4-bis[2-(5-phenyloxzoly)]benzene, and xylenes to 1000 ml.

and the test substrate. After incubation for 30 min at 37°, reactions were stopped by heating at 100° for 3 min; the assay mixtures were then clarified by centrifugation (8000 x g for 1 min), freeze-dried, and analyzed by chromatography. Apyrase reaction mixtures contained the following in a final volume of 100 μ l: 100 mM potassium succinate buffer (pH 6.5), 1.8 mM CaCl_2 , 1 unit of apyrase and the test substrate. After incubation for 30 min at 37°, assay reactions were stopped by heating at 100° for 3 min, and the mixtures were analyzed by chromatography.

F. Nucleoside Uptake Studies

1. RPMI 6410 Cells.

Rates of uptake of nucleosides by RPMI 6410 cells were determined as follows. Cell suspensions in RPMI medium 1640 with 5% dialyzed fetal calf serum were used: uptake intervals were initiated by the addition of ^3H -nucleoside and terminated by transferring 1.0 ml samples of the assay mixtures into 40 ml of cold 0.15 M NaCl; after centrifugation (500 x g for 3 min at 4°), cell pellets were washed once with 20 ml of cold 0.15 M NaCl, dissolved in 0.2 ml of 0.5 N KOH and assayed for radioactivity by liquid scintillation counting.

2. HeLa Cells.

Nucleoside uptake by HeLa cell monolayers was assayed as follows: Cell sheets were incubated for timed intervals at 37° in MEM-T (or transport) medium (MEM with 10% calf serum, 2 mM HEPES buffer, pH 7.4, and 1.8 mM calcium salts)

containing ^3H -nucleoside; to terminate the uptake interval, medium was removed by suction and cell sheets were flooded with 60 ml of cold 0.15 M NaCl. After thorough drainage, the cell sheets were dissolved in 1.5 ml of 0.5 N KOH and assayed for radioactivity as described in Section D above.

G. Cell Cycle Studies

Progress through the cell cycle by synchronous cultures of RPMI 6410 cells was evaluated by the following criteria:

(a) cell concentration, (b) rate of incorporation of ^3H -dThd into acid-insoluble material, (c) mitotic index (M.I.), and (d) flow microfluorimetry.

In measuring incorporation of dThd into acid-insoluble material, cell suspensions consisting of 3 to 6 x 10⁵ cells in 1.1 ml of growth medium containing 18.2 nM [methyl- ^3H]dThd (1 μCi) were incubated at 37° in 10% CO₂-air for 10 min. After addition of 10 ml of cold 5% TCA, cells were collected on 1.2 μ nitrocellulose filters which were then rinsed with three 10-ml portions of 5% TCA and assayed for radioactivity by the combustion-liquid scintillation method.

For M.I. determination, 0.6 to 1.2 x 10⁵ cells were centrifuged onto slides, exposed to Wright's stain (250 mg Wright's stain in 100 ml methanol) for 4 min, then to 5 mM KH₂PO₄ (pH 6.8) for 8 min and rinsed with water. At least 2000 cells were scored for each M.I. determination.

Changes in the frequency distribution of DNA content of

cell populations were appraised by flow microfluorimetry*: cells were collected by centrifugation and stained for 15 min with propidium iodide (0.05 mg/ml) in 0.1% sodium citrate. The cellular fluorescence intensities were recorded on a Bio/Physics Model 4800A cytofluorograph equipped with a Bio/Physics Model 2100 pulse-height analyzer (Bio/Physics Systems Inc., Mahopac, N.Y.).

H. Preparation and Assay of Cellular Extracts Containing Deoxycytidine Kinase and Deoxythymidine Kinase

1. RPMI 6410 Cells.

Cell extracts for assay of dThd and dCyd kinase activities were prepared as follows: 10^8 RPMI 6410 cells in growth medium were cooled, collected by centrifugation ($500 \times g$ for 3 min at 4°) and rinsed with 100 ml cold 0.15 M NaCl. The cells were resuspended in 1 ml of cold extraction buffer (0.1 M Tris, pH 7.5, 10% (v/v) glycerol, 0.15 M NaCl, 2 mM dithioerythritol, 20 mM 6-aminocaproic acid), frozen and thawed 4 times in an acetone-Dry Ice mixture and homogenized at 4° with 30 strokes of a Dounce homogenizer. The mixture was then centrifuged ($10,000 \times g$ at 4° for 10 min) and the supernatant reserved for assay of kinase activities. Kinase assay mixtures contained the following in a final volume of 0.05 ml: 0.1 M Tris-HCl (pH 7.5), 1.9 mM ATP, 6 mM $MgCl_2$,

*Flow microfluorimetric studies were performed in the laboratory of Dr. L.W. Brox.

0.1 mg ovalbumin, 6 mM creatine phosphate, 8 mM NaF, 2 mM dithioerythritol, 1 unit of creatine kinase, cell extract and varying concentrations of radioactive substrate (28). To end assay intervals, 25 μ l samples of the assay mixtures were transferred onto DEAE cellulose paper squares (2.5 x 2.5 cm) which were immersed individually into 10 ml of 0.1 mM ammonium formate (pH 3.4). Each square was then rinsed (10 min each rinse) 3 times with 10 ml of the latter, once with 10 ml water and once with 10 ml 95% ethanol and assayed for ^3H by the combustion-liquid scintillation procedure. "Product" formation in mixtures containing heated cellular extract (100°, 3 min) represented only 50-60 cpm above background, irrespective of the specific activity of labelled substrate. The 10,000 x g sediments (see above) did not contain measurable dThd or dCyd kinase activities.

2. HeLa Cells.

Cell extracts for assay of dThd and dCyd kinase activities were prepared from individual monolayer cultures (10^6 cells) as follows: growth medium was removed, cells were rinsed once with 60 ml of cold 0.15 M NaCl and 1.2 ml of cold extraction buffer (described above) was added. After 1 min at 4°, the cell sheets were frozen and thawed 4 times in an acetone-Dry Ice mixture, and the resulting solutions were centrifuged at 10,000 x g for 10 min at 4°. The supernatants were reserved for assay of kinase activities as described above.

3. Affinity Chromatography of Deoxycytidine Kinase. Cheng et al. (28) have purified dCyd kinase by affinity chromatography using dThd-linked Sepharose; elution of dCyd kinase was accomplished with increasing ionic strength and dThd concentrations. In the present study, dCyd kinase affinity gels were prepared by Dr. T.P. Lynch of this laboratory by the procedure of Lee and Cheng (87). The content of the derivatized gel was determined by spectrophotometry after hydrolysis of a sample of dried gel: 9.19 μ moles of dThd were bound per g of dried gel.

I. Synthesis of AraCDPcholine

The method employed by Kennedy (74) for the synthesis of cytidine-5'-diphosphate choline (CDPcholine) was adapted to the synthesis of 1- β -D-arabinofuranosylcytosine-5'-diphosphate choline [araCDPcholine] (84; see Appendix B).

J. Miscellaneous Procedures

Cell concentrations were determined with an electronic particle counter. Cell volumes were obtained from cell volume distributions measured with an electronic cell counter coupled to a multichannel particle size analyzer (Coulter Electronics, Hialeah, Fl.) calibrated with polystyrene microspheres.

All commercially obtained radiolabelled compounds were analyzed for radiochemical purity by thin layer chromatography (Table 1). Where necessary, radiolabelled compounds

were purified by chromatography on 250 μ thin layers of cellulose (Cellulose MN300, Machery, Nagel and Co., Germany) using volatile solvent systems.

The specific activity of each radiolabelled chemical was determined under the same conditions used for the assay of its radioactivity in experimental samples.

Studies on the binding of the nucleoside transport inhibitor, 6[(4-nitrobenzyl)thio]-9- β -D-arabinofuranosyl-purine (NBMPA), to RPMI 6410 cells were performed as described previously using HeLa cells grown in suspension culture (85; see Appendix A).

K. Chemotherapy and Murine Toxicity Experiments

BDF₁ hybrid mice (female C57Bl/10J x DBA/2J, F₁ generation) and BALB/c mice were obtained from the Health Sciences Small Animal Program, University of Alberta. Leukemia L1210 cells were maintained by the weekly passage of 10^5 cells in DBA/2J mice. In individual chemotherapy and toxicity experiments, mouse weights were within ± 1.5 g. Drugs were dissolved in 0.15 M NaCl and were administered by i.p. injections which were proportional to 0.2 ml per 20 g body weight at the time of implantation, unless specified otherwise.

III. Inhibition of RPMI 6410 Cell Proliferation

A. Introduction

Experiments presented in this segment studied the inhibition of RPMI 6410 cell proliferation by araC, DU, PF, HU and dThd. RPMI 6410 cells were also cultured in the presence of araC and DU together; such combinations were synergistic with respect to toxicity. The influence of DU on araC metabolism was assessed in preliminary experiments which studied the effect of DU pretreatment of RPMI 6410 cells on the toxicity of subsequent exposures to araC; pretreatment with DU "sensitized" these cells to the toxic effects of araC.

B. Results

Fig. 3 illustrates the concentration-dependence of the inhibition of RPMI 6410 cell proliferation by araC, DU, PF, HU and dThd. IC_{50}^* values for these drugs were: araC, 0.29 μ M; PF, 0.21 μ M; DU, 4.8 μ M; HU, 113 μ M. In medium containing 1 mM dThd, RPMI 6410 cells proliferated at 35% of the control rate; this inhibition was reversed by the addition of 5 μ M dCyd, as in earlier experiments with L5178Y cells (107).

In the experiment of Fig. 4, RPMI 6410 cells were exposed under culture conditions for 24 hr to an araC-DU

*Concentrations of inhibitor that reduced the number of cell doublings during 48 to 50 hr of continuous exposure to 50% of that observed in untreated cultures.

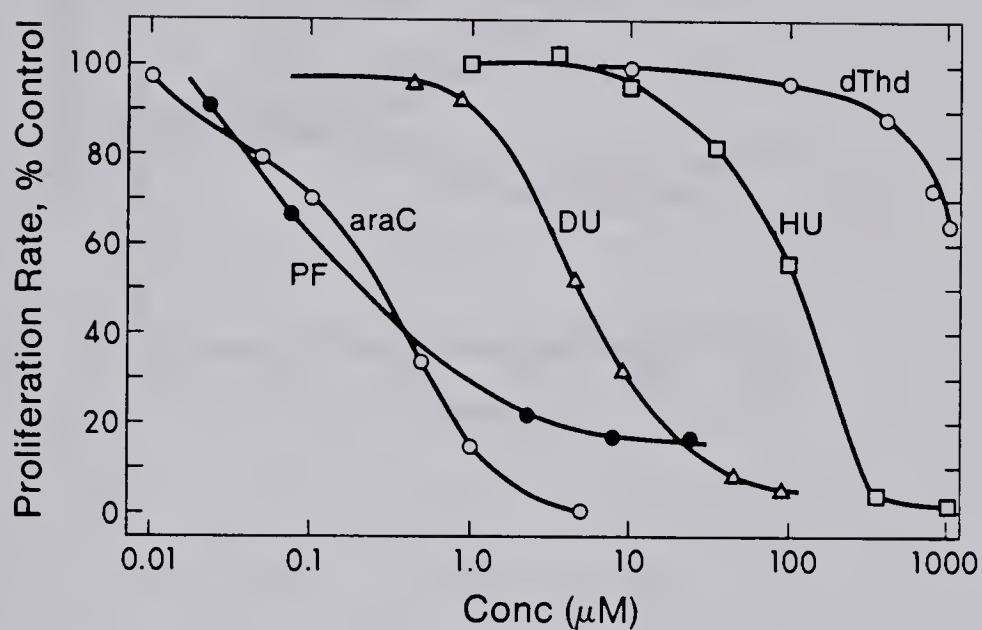


Figure 3. Inhibition of RPMI 6410 cell proliferation. Cells were cultured for 48 hr in medium containing the indicated concentrations of araC, PF, DU, HU and dThd. The number of cell doublings during this interval was expressed as a percentage of corresponding values (2.4 to 2.9 doublings) for cells cultured in the absence of drugs.

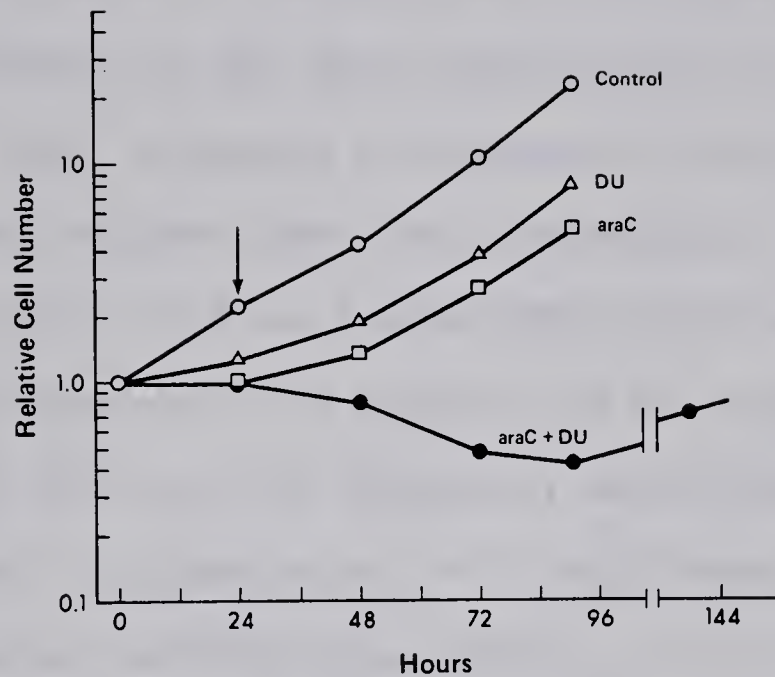


Figure 4. Synergism in the cytotoxicity of DU and araC toward RPMI 6410 cells. Cells were cultured without additives (control) or in medium containing 9 μ M DU, 3 μ M araC or both for 24 hr. At that time (arrow), cells were collected by centrifugation at 150 g for 5 min, washed once with warmed, drug-free medium and resuspended in growth medium for continued culture.

combination prior to culture in drug-free medium. It is seen that when cells exposed to araC or DU alone were transferred to drug-free medium, growth rates were close to those in untreated cultures. However, proliferation rates of those cells exposed to the drug combination did not recover, even after 140 hr. Attempts to determine viability of these cells by cloning methods were not successful.

The experiment of Fig. 5 with RPMI 6410 cells demonstrated the effect of exposure to DU prior to 4 hr intervals of incubation with araC and DU together; drug effects were apparent by their influence on cell proliferation rates following transfer to drug-free medium. It is evident (Fig. 5) that the interval of DU pretreatment enhanced the toxicity of araC. Culture in the presence of DU alone for intervals up to 24 hr had little effect on the proliferation rates of RPMI 6410 cells following transfer to drug-free medium (Fig. 4).

C. Discussion

The growth-inhibitory effects of araC and DU toward RPMI 6410 cells were enhanced synergistically when these agents were present together (Fig. 4); the surviving fraction* of cells treated for 24 hr with both agents was approximately one-tenth of that expected from the sum of the cytotoxic

*Surviving fraction was obtained by back extrapolation of cell growth curves (as in Fig. 4), assuming control growth rate in drug treated cultures 112 hr after transfer to growth medium without additives.

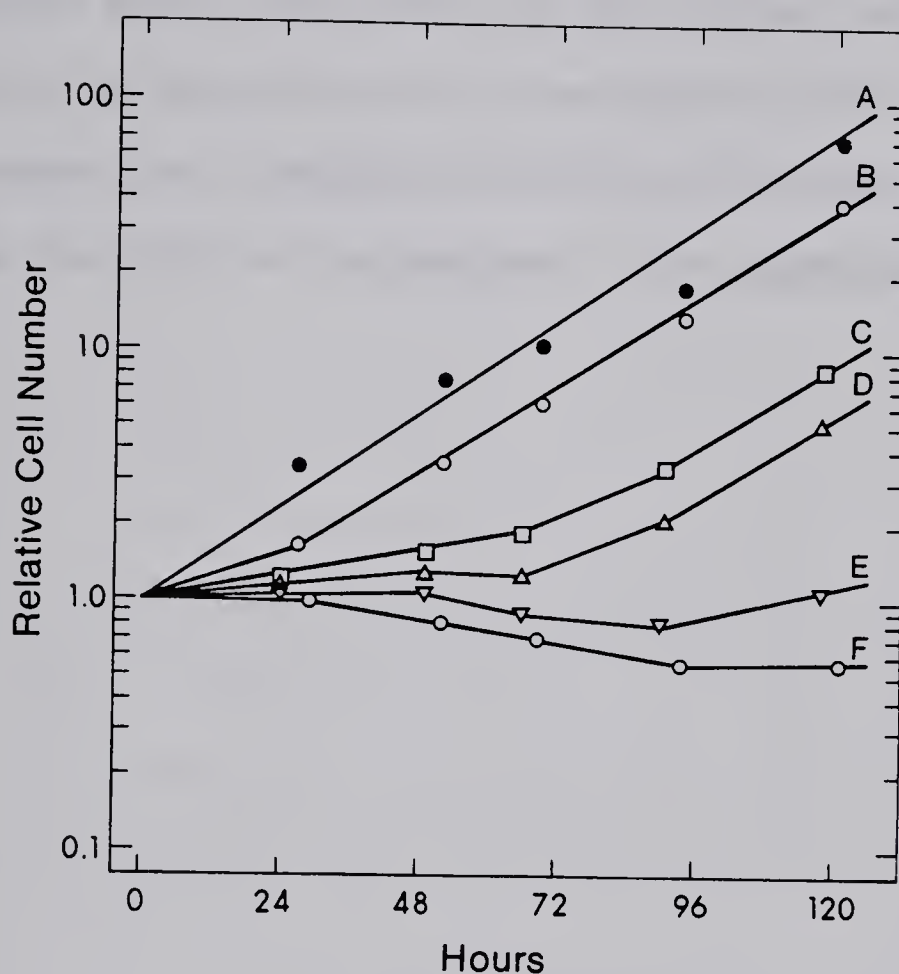


Figure 5. Influence of DU pretreatment on the toxicity of araC toward RPMI 6410 cells. Cells were cultured in the presence of 10 μ M DU for intervals of 0 (B), 2 (C), 4 (D), 6 (E), or 18 hr (F), further incubated for 4 hr in medium containing 10 μ M DU and 10 μ M araC, and then resuspended (time zero) in drug-free medium for continued culture. Plot A represents cell proliferation in the absence of additives.

effects of the separate agents. Furthermore, the toxicity of araC was greatly enhanced by prior exposure to 10 μ M DU, especially for intervals of DU-exposure greater than 6 hr (Fig. 5). These results suggest that a metabolic effect of DU "sensitized" RPMI 6410 cells to the lethal action of araC. Studies by Brenckman and coworkers (1973) have shown that pretreatment of lymphoma L5178Y cells with 6-azauridine enhanced the toxicity of subsequent 2 hr exposures to araC.

IV. Metabolism of AraC

A. Metabolism of AraC in RPMI 6410 Cells in the Presence or Absence of DU

1. Introduction.

We attempted to find a biochemical explanation for the influence of DU pretreatment on the cytotoxicity of araC toward RPMI 6410 cells (see Chapter III). The metabolism of araC was studied in the presence or absence of DU and these investigations produced two main conclusions: (i) the anabolism of araC was stimulated several-fold in RPMI 6410 cells cultured in the presence of DU, and (ii) araCDPcholine and araCDPethanolamine, hitherto unrecognized anabolites of araC, were found in RPMI 6410 cells. A portion of these observations have been published (84; see Appendix B) and is dealt with only summarily in this presentation.

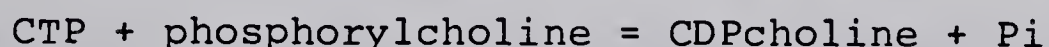
2. Results.

i. Summary of Appendix B. When incubated with araC, RPMI 6410 cells formed the hitherto unrecognized metabolites araCDPcholine and araCDPethanolamine. AraCDPethanolamine was consistently a minor metabolite of araC relative to araCDPcholine. The latter compound was characterized by (a) chromatographic behavior on thin layers of PEI-cellulose and on columns of Dowex 1-formate and Dowex 50-⁺H, (b) chemical and enzymatic hydrolysis, (c) phosphorus content and (d) incorporation of [5-³H]araC and [methyl-¹⁴C]choline.

To better understand the basis of the "sensitization" by

DU of RPMI 6410 cells to the lethal action of araC (see Chapter III), the anabolism of 3 μ M araC was studied in the presence of 9 μ M DU; after a 24 hr interval of culture, cellular concentrations of araCTP and araCDPcholine (the two major anabolites of araC) were 5- and 15-fold higher, respectively, than in the absence of DU. In the presence of DU, cellular concentrations of araCTP and araCDPcholine were maximal after 14 hr of incubation; at that time, cellular levels of araCDPcholine were about 2-fold greater than those of araCTP. In the absence of DU, cellular concentrations of araCTP peaked within 3 hr and fell thereafter; the content of araCDPcholine increased gradually until, after 22 hr of incubation, cellular levels of both metabolites were similar.

In tissues, CDPcholine is formed by a reversible reaction catalyzed by phosphorylcholine cytidylyltransferase [EC 2.7.7. 15] (76):



This enzyme also accepts dCTP as substrate (75) and the product, dCDPcholine, has been demonstrated in various tissues (75,143,154). As would be expected from the above scheme, the appearance of araCTP as a metabolite of araC in RPMI 6410 cells preceded that of araCDPcholine, whether DU was present or absent.

The effect of nucleosides on the DU enhancement of araC anabolism was studied: the stimulatory influence of DU was essentially abolished in the presence of dCyd and markedly reduced by Cyd and Urd. In the presence of Cyd, the ratio

of araCTP to araCDPcholine was increased, possibly suggesting competition between CTP and araCTP for phosphorylcholine cytidylyltransferase.

In attempts to provide a basis for the stimulatory influence of DU on araC anabolism, the effect of culture with DU on nucleotide concentrations in RPMI 6410 cells was investigated. Cellular concentrations of Cyd phosphates, and presumably of dCyd phosphates, were depleted in cells incubated in the presence of 9 μ M DU for intervals greater than 6 hr. These observations substantiate earlier results obtained by Brockman and coworkers (19) using leukemia L1210 cells.

To further probe the basis by which DU "sensitized" RPMI 6410 cells to araC cytotoxicity, the influence of the duration of DU exposure on the ability of cells to take up araC in short term assays was examined: initial rates of araC uptake were increased about 7-fold by a 12-hr interval of culture in the presence of DU (Chart 5, Appendix B). Cells increased in volume during exposure to DU; however, increases in araC uptake were not directly related to the volume increases.

ii. Additional Findings. Fig. 6 shows that the DU-enhanced uptake of araC was inhibited by NBMPR, a potent inhibitor of nucleoside transport (see Chapter I, Section F). This result implies that the entry of araC into the lymphoid cells was mediated by the nucleoside transport mechanism. This finding was applied to the experiment of

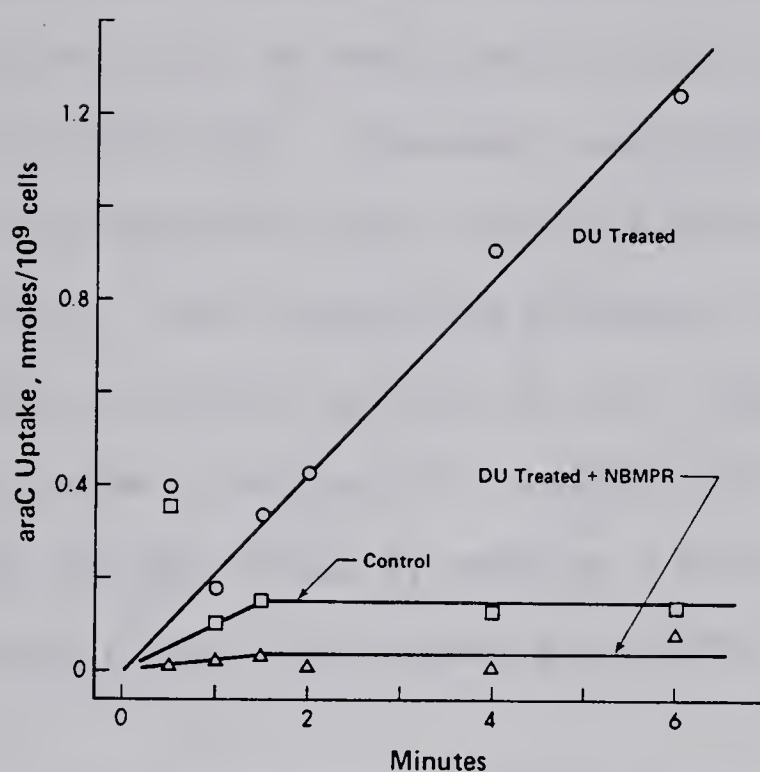


Figure 6. Inhibition of DU-enhanced araC uptake by NBMPR, an inhibitor of nucleoside transport. After culture for 12 hr in the absence (control, □) and presence (○, △) of 9 μ M DU, RPMI 6410 cells were washed in warmed, DU-free medium and assayed in duplicate for their ability to take up araC in the absence (○, □) and presence (△) of 5 μ M NBMPR.

Fig. 7 which demonstrated that cells were partially "protected" by NBMPR against growth inhibition by both araC and DU.

The effect of DU pretreatment on the uptake of dCyd and dThd was also studied (Fig. 8); the uptake of $[2\text{-}^{14}\text{C}]\text{dCyd}$ by RPMI 6410 cells was enhanced following 12 hr of culture in DU-containing medium, as was that of araC (Fig. 6 above and Chart 5 in Appendix B). However, reduction in the uptake of dThd was apparent when uptake intervals were longer than 15 min. This reduction probably reflected inhibition of DNA synthesis by DU, as will be apparent in subsequent discussion (Chapter VI, Section B). The inhibition of dThd uptake (Fig. 8) may be a consequence of the inhibition of dThd kinase by accumulated dTTP (115).

B. Formation of AraCDPcholine in Neoplastic and Normal Cells in the Presence or Absence of DU.

1. Introduction.

In this segment of our study, the formation of araCDPcholine and araCDPethanolamine as metabolites of araC was established as a general phenomenon; the influence of DU on araC anabolism in various types of cells and tissues was also evaluated. A portion of these observations have been published (86; see Appendix C); a summary of Appendix C follows.

2. Results.

i. Summary of Appendix C. AraCDPcholine was formed from araC during in vitro incubation of (a) leukemic leukocytes from patients with acute myelogenous

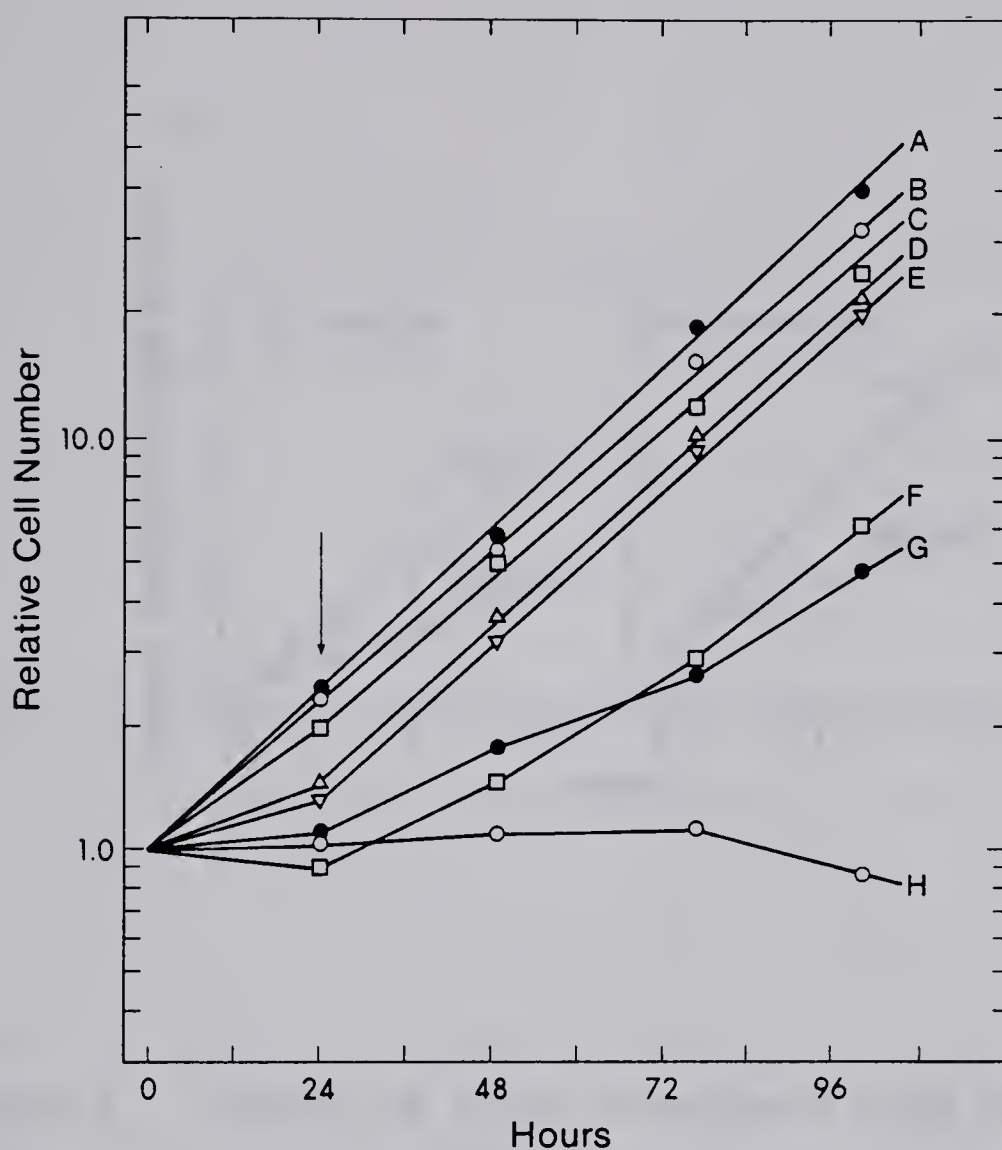


Figure 7. NBMPR protection of RPMI 6410 cells against growth inhibition by araC and DU. Cells were cultured without additives (A) or in the presence of 5 μ M NBMPR (B), 9 μ M DU + NBMPR (C), 3 μ M araC + NBMPR (D), 3 μ M araC + 9 μ M DU + NBMPR (E), 9 μ M DU (F), 3 μ M araC (G) or 3 μ M araC + 9 μ M DU (H) for 24 hr. At that time (arrow), cells were collected by centrifugation and culture was resumed as described in Fig. 4.

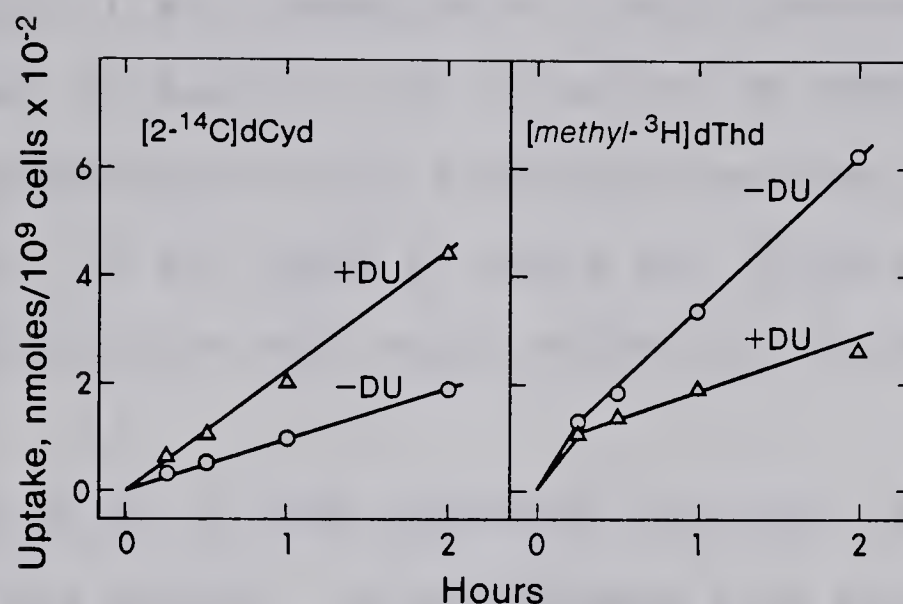


Figure 8. Effect of prior treatment with DU on the uptake of dCyd and dThd by RPMI 6410 cells. Cells were cultured in the presence or absence of 10 μ M DU for 12 hr prior to the addition of [2-¹⁴C]dCyd or [methyl-³H]dThd to obtain final concentrations of 5 μ M (0.2 μ Ci/ml) and 1 μ M (0.5 μ Ci/ml), respectively. After the intervals indicated, total cellular radioactivity was determined as described in Chapter II, Section D.

leukemia (AML), and (b) cultured cells of several lines, including human nonleukemic lymphocytes (LS cells), mouse lymphoma L5178Y and HeLa. When leukemic leukocytes from AML patient W.L. were incubated in vitro with araC, concentrations of araCTP were maximal after 8 hr of incubation (see Chart 1, Appendix C) and formation of araCTP preceded that of araCDPcholine, in analogy with formation of CDPcholine by the action of phosphorylcholine cytidylyltransferase, as described above. In all types of cells and tissues examined, araCTP and araCDPcholine were major anabolites derived from araC.

The effect of DU on araC anabolism differed in the various cell lines tested. In myeloblasts from patients W.L. (see Chart 1 in Appendix C) or M.W.* (Table 2, Appendix C), araC anabolism was not influenced by exposure to 8-10 μM DU, but that in myeloblasts from patient D.L.* was inhibited by DU. Also, the cellular content of Cyt phosphates in myeloblasts from patient W.L. was not affected by culture in the presence of 9 μM DU for 24 hr, in contrast to the profound reduction which occurred in RPMI 6410 cells. AraC anabolism in lymphoma L5178Y cells was unchanged in the presence of 2.7 μM DU. In LS cells**, the anabolism of 0.3

*The labelling indices (determined by Dr. L.W. Brox) of myeloblasts from patients M.W. and D.L. were 6.0% and 0.5%, respectively.

**With LS cells, IC_{50} values for 48 hr intervals of culture in medium containing araC or DU were 0.3 and 12 μM , respectively; with HeLa cells, corresponding values were 0.1 and 2.6 μM .

μM araC was enhanced 2-3 fold in the presence of 8 μM DU, whereas that of 3 μM araC was enhanced only marginally.

In HeLa cells, 10 μM did not influence araC anabolism. Treatment of these cells with 10 μM DU for 6 hr affected cellular nucleotide concentrations as follows: levels of ATP, GTP and UPT were enhanced 120%, 127% and 135%, respectively, above those in untreated cells. Concentrations of CTP were reduced to 46% of control levels with 4 hr of exposure to 10 μM DU but did not decline further. Concentrations of ATP and CTP in untreated HeLa cells were 3980 and 790 nmoles/ 10^9 cells, respectively. The failure of 10 μM DU to enhance araC anabolism in HeLa cells, as in other types of cells, may be related to the partial depletion of Cyd nucleotides under the conditions of DU exposure employed. This view is supported by our finding that culture in the presence of 100 μM DU depleted CTP concentrations in HeLa cells within 4 hr; araC anabolism was enhanced under those circumstances (Chapter V). Further, 100 μM DU enhanced the anabolism of dCyd and araC in Novikoff hepatoma cells (133).

Prior treatment of leukemic mice with DU (60 mg/kg) enhanced 3-fold the in vivo anabolism of araC in leukemia L1210 cells; ascites cells were collected 2 hr after the i.p. administration of araC (15 mg/kg). In the absence of DU, araCTP and araCDPcholine accounted for 73 and 8% of acid-soluble radioactivity, respectively; in the presence of DU, corresponding fractions were 59 and 23%. Brockman and

coworkers (19) demonstrated that administration of DU (60 mg/kg) depleted cellular Cyd and dCyd phosphates in leukemia L1210 cells in vivo.

Since liver is rich in CDPcholine and phosphorylcholine cytidylyltransferase (76), we determined the metabolites of araC in the liver of mice 1.5 hr after the i.p. administration of araC: the summed content of araCDPcholine and araCDPethanolamine was about 20-fold greater than that of araCTP, suggesting rapid conversion of the latter to the former in liver. Pretreatment with DU did not significantly alter the concentration of araC metabolites in liver in vivo.

ii. Additional Findings. The data of Table 2 demonstrate the formation of araC metabolites in peripheral myeloblasts from a patient (E.H.) with chronic myelogenous leukemia. In these cells, DU enhanced araC anabolism 1.3- to 1.7-fold; further, the proportion of the acid soluble radioactivity associated with araCDPcholine and araCDPethanolamine was increased when DU was present, with corresponding decreases of that associated with araCTP. The labelling index of myeloblasts from patient E.H. was 8.5%.

C. Discussion.

The instances of araC conversion to the anabolites araCDPcholine and araCDPethanolamine reported here would appear to establish this conversion as a general phenomenon. Thus, araCTP evidently serves as substrate in the reactions catalyzed by phosphorylcholine cytidylyltransferase and phosphoryl-

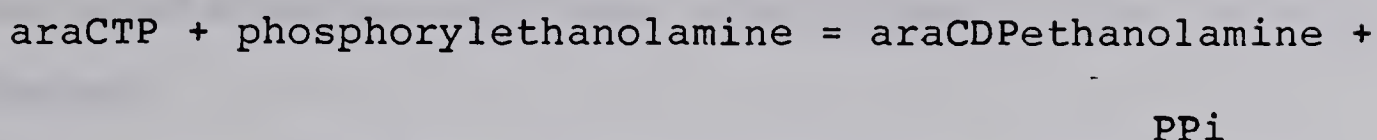
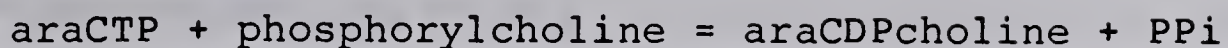
TABLE 2

Metabolites of araC in CML myeloblasts

Myeloblasts from CML patient E.H. were incubated for 24 hr in medium containing [5,6-³H]araC and DU as indicated. Perchloric acid extracts of the cells were analyzed by TLC.

Conc (M)		Total araC metabolites (nmole/10 ⁹ cells)	Distribution of radioactivity (% of total)							
araC	DU		araCTP	araCDP	araCMP	araCDPch	araCDPet	araC+ araU	araUTP	ara- UMP
9	9	44.5	27.3	9.1	0.8	63.4	13.9	4.9	5.4	0.5
9	0	33.1	52.7	5.3	8.1	35.1	6.2	12.1	3.4	7.7
3	9	23.3	35.7	10.9	0.8	48.7	18.6	9.4	4.0	1.2
3	0	15.4	55.3	4.7	1.3	31.2	11.2	8.2	3.2	12.6
0.3	9	5.2	35.4	5.9	0.8	38.1	24.3	17.5	1.9	3.4
0.3	0	3.0	60.4	4.6	0.7	28.0	13.9	7.6	1.6	8.9

ethanolamine cytidylyltransferase:



The formation of these araC anabolites was previously not recognized despite many studies evaluating araC metabolism (39), possibly because araC anabolites were determined at (1) short intervals (less than 1 hr) after the provision of araC (e.g. ref 145) or (2) by means of chromatographic isolation of isotopic araC metabolites from tissue extracts using carrier techniques which detect only those metabolites which the investigator presumes will be present (e.g. ref. 31).

In order to assure recognition of the principal cellular metabolites of [³H]araC, these studies employed techniques which (1) accounted for greater than 95% of the acid-soluble ³H-content of the cellular acid-soluble fraction, (2) included short and long term intervals of drug exposure, (3) established metabolite identification in at least two TLC solvent systems and (d) studied araC metabolism in a variety of cell types.

At present, little is known about the possible contributions of araCDPcholine and araCDPethanolamine formation to araC cytotoxicity. Since the phosphorylcholine cytidylyltransferase reaction is reversible (76), cellular pools of araCDPcholine may sustain those of araCTP. AraCDPcholine was the major cellular anabolite of araC (Chart 4, Appendix B) under the conditions of the synergistic toxicity of the araC-DU combination toward RPMI 6410 cells (Fig. 4, Chapter

III). Both CDPcholine (76) and dCDPcholine (75,142) donate the phosphorylcholine moiety in phosphatidylcholine synthesis; whether araCDPcholine will also participate in this reaction is unknown.

DU enhanced the anabolism of araC in RPMI 6410 cells and LS cells in vitro and in leukemia L1210 cells in vivo; araC uptake was similarly enhanced in RPMI 6410 cells pretreated with DU. We suggest that the enhancement of araC anabolism by DU is (1) the basis of enhancement of cytotoxicity which resulted when RPMI 6410 cells were treated with araC and DU (Fig. 4, Chapter III), (2) the basis for the increased sensitivity to araC in RPMI 6410 cells after pretreatment with DU (Fig. 5, Chapter III), and (3) a consequence of the DU-induced reduction of the cellular concentrations of Cyd phosphates (and presumably of dCyd phosphates) in RPMI 6410 cells, as demonstrated in Table 2, Appendix B. The latter idea is supported by our demonstration that the provision of dCyd to RPMI 6410 cultures abolished the enhancement of araC anabolism by DU (Table 5, Appendix B).

V. Effects of dThd, PF and HU on the Metabolism of AraC

A. Introduction

In the preceding chapter, it was demonstrated that DU increased araC anabolism in RPMI 6410 cells and LS cells in culture and leukemia L1210 cells in vivo. It was suggested that this enhancement was a consequence of the depletion of cellular dCyd phosphates. The present chapter describes the effect on araC anabolism in cultured cells of incubation with (1) dThd or PF, which would be expected to reduce cellular levels of dCyd phosphates and (2) HU, which has been shown to enhance dCyd uptake (132) and, for comparison, examples of the influence of DU are presented. As well, the influence of the above-mentioned agents on the formation of araCDPcholine and araCDPethanolamine is described. These studies were performed with RPMI 6410 cells, HeLa cells, lymphoma L5178Y cells and human leukemic leukocytes.

B. Results

1. RPMI 6410 Cells.

Table 3 compares the effects of 1 mM dThd, 10 μ M DU and 200 μ M HU on the uptake of araC and incorporation of the latter into the cellular acid-insoluble fraction during intervals up to 6 hr. After 0.5 and 6 hr in the presence of araC and HU, cellular concentrations of acid-soluble araC metabolites were enhanced 2.2 and 8.5-fold, respectively, above those attained in the presence of araC alone. The effects of DU and dThd on araC uptake were expressed later and were smaller

TABLE 3

Drug-enhanced uptake of araC in RPMI 6410 cells

Cells were cultured in medium containing 3 μM [5,6- ^3H]-araC (1 $\mu\text{Ci/ml}$) without additives (control) or with 1 mM dThd, 10 μM DU or 200 μM HU; after the intervals indicated, acid-soluble and acid-insoluble fractions of the cells were assayed for radioactivity.

Time (hr)	Acid-soluble metabolites (nmoles/ 10^9 cells)			
	Control	+dThd	+DU	+HU
0.5	23.25	30.60	29.44	50.59
1	35.36	47.15	41.93	85.66
2	46.26	79.59	59.73	144.42
4	43.47	109.76	94.12	228.13
6	34.61	123.86	144.76	296.67

Time (hr)	Acid-insoluble metabolites (nmoles/ 10^9 cells)			
	Control	+dThd	+DU	+HU
0.5	0.19	0.13	0.08	0.12
1	0.27	0.41	0.35	0.41
2	0.47	0.60	0.45	0.35
4	0.89	1.20	0.94	0.90
6	1.16	1.47	1.33	1.27

than those of HU. In contrast, dThd, DU and HU did not influence significantly the incorporation of araC into the acid-insoluble fraction (Table 3). Two similar experiments yielded results much like those of Table 3. These observations agree with those reported recently by Plagemann and coworkers (133).

Cellular concentrations of araC metabolites formed during culture of RPMI 6410 cells in the presence of dThd or PF are reported in Fig. 9. Cellular concentrations of araCTP and araCDPcholine were increased 8.7- and 6.0-fold, respectively, after 13 hr of culture when dThd was present. The ^3H -content of the acid-soluble fraction had increased 1.5-fold after 13 hr in the presence of 1 μM PF, a reflection of 1.4- and 6.6-fold increases of the content of araCTP and araCDPcholine, respectively (Fig. 9). Thus, the stimulatory effect of PF on araC anabolism in RPMI 6410 cells was expressed mainly on the formation of araCDPcholine. It is noted that in the presence of 1 μM PF the proliferation of RPMI 6410 cells was inhibited by approximately 70% (Fig. 3).

Table 4 lists cellular concentrations of araC metabolites formed in RPMI 6410 cells after incubation in the presence or absence of 200 μM HU. The HU-enhanced uptake of araC (see Table 3) was attributed mainly to increased cellular concentrations of araCTP and araCDPcholine (Table 4).

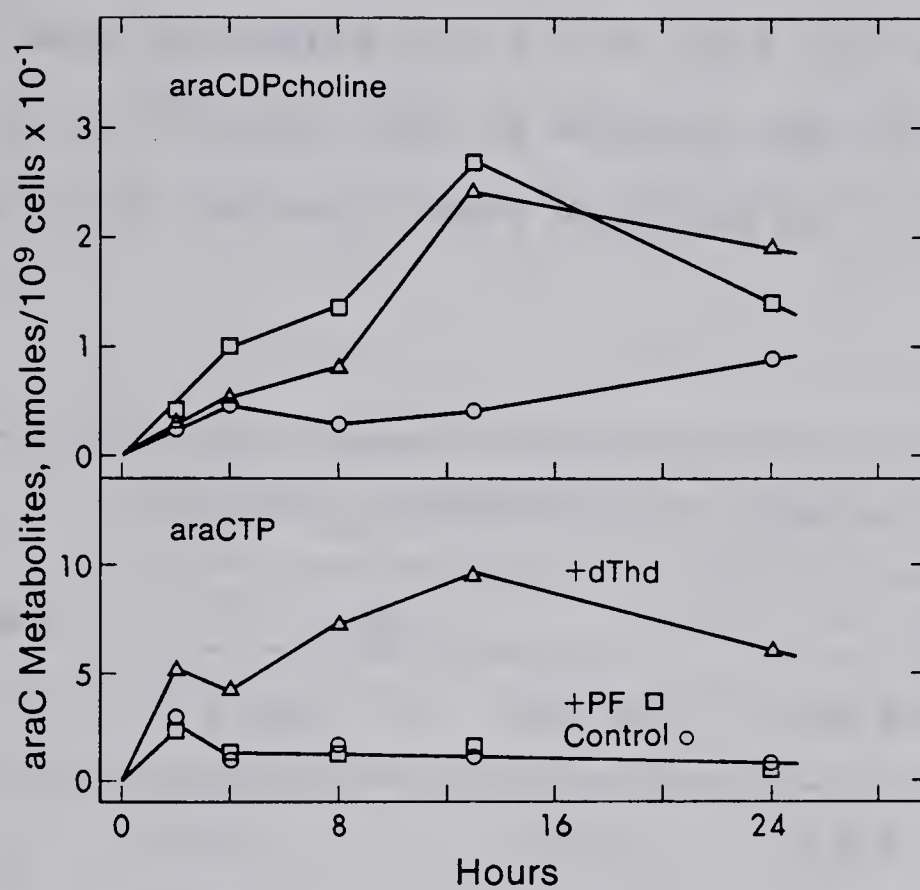


Figure 9. Effects of dThd and PF on the formation of araC metabolites in RPMI 6410 cells. Cells were cultured in medium containing 3 μM [5,6- ^3H]araC with 1 mM dThd (Δ), 1 μM PF (\square) or without additions (control, \circ). At the indicated intervals, perchloric acid extracts of the cells were chromatographed on PEI-cellulose thin layers using solvent 1.

TABLE 4

Metabolites of araC in HU-treated RPMI 6410 cells

Cells were incubated for 5.5 or 24 hr in medium containing 3 μ M [5-³H]araC with or without 200 μ M HU; perchloric acid extracts of the cells were analyzed by TLC using solvent 1.

Metabolite	Cellular concentration (nmoles/10 ⁹ cells)			
	-HU		+HU	
	5.5 hr	24 hr	5.5 hr	24 hr
AraCTP	23.0	10.7	119.5	126.2
AraCDP	2.4	0.8	11.6	15.9
AraCMP	1.0	0.5	3.5	5.7
AraCDPcholine	7.9	6.7	20.2	61.0
AraC	3.8	4.1	5.6	4.7
Acid-insoluble	1.7	10.9	1.2	4.1

However, the proportion of the acid-soluble radioactivity present in the form of araCDPcholine was smaller than that following following culture in the presence of DU (Chart 4, Appendix B).

2. HeLa Cells.

The enhancement of araC uptake in HeLa cell monolayers by the addition of 1 mM dThd*, 200 μ M HU or 100 μ M DU* is shown in Fig. 10. In accordance with results obtained above with RPMI 6410 cells, HU effects were expressed earlier and were more pronounced than those of either dThd or DU. DU, at a concentration of 10 μ M, did not influence araC anabolism under the conditions of Fig. 10 (data not shown).

Fig. 11 illustrates the influence of dThd and PF on the formation of araC metabolites in HeLa cells. The dThd-enhanced uptake of araC by these cells (Fig. 10) was accounted for by increases of the cellular concentrations of araCTP and araCDPcholine. However, the effect of PF was restricted to the anabolite araCDPcholine, the concentration of which was stimulated 2-fold after culture for 7 hr in the presence of PF.

3. L5178Y Cells.

Fig. 12 illustrates the time course of araC metabolite formation in lymphoma L5178Y cells cultured in the presence

*IC₅₀ values for 48 hr intervals of culture in the presence of dThd and DU were 2.2 mM and 2.6 μ M, respectively.

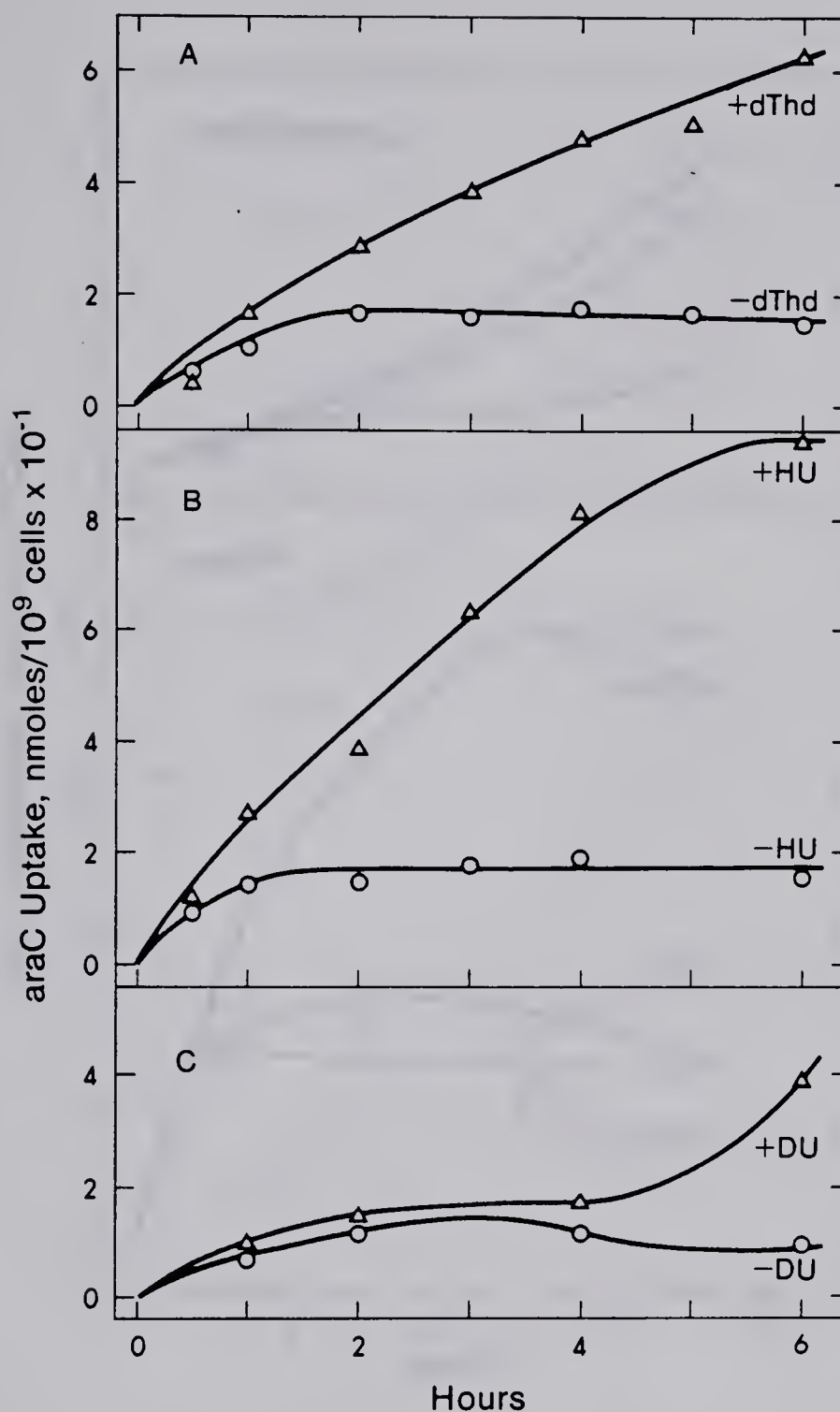


Figure 10. Effects of dThd, HU and DU on araC uptake by HeLa cells. Cells in monolayer culture were incubated in medium containing 0.6 μM [5,6-³H]araC with or without 1 mM dThd (A), 200 μM HU (B) or 100 μM DU (C). After the intervals indicated, the cell sheets were dissolved and assayed for radioactivity; points are averages of araC incorporation in 3 monolayers. Confirmatory experiments yielded similar results.

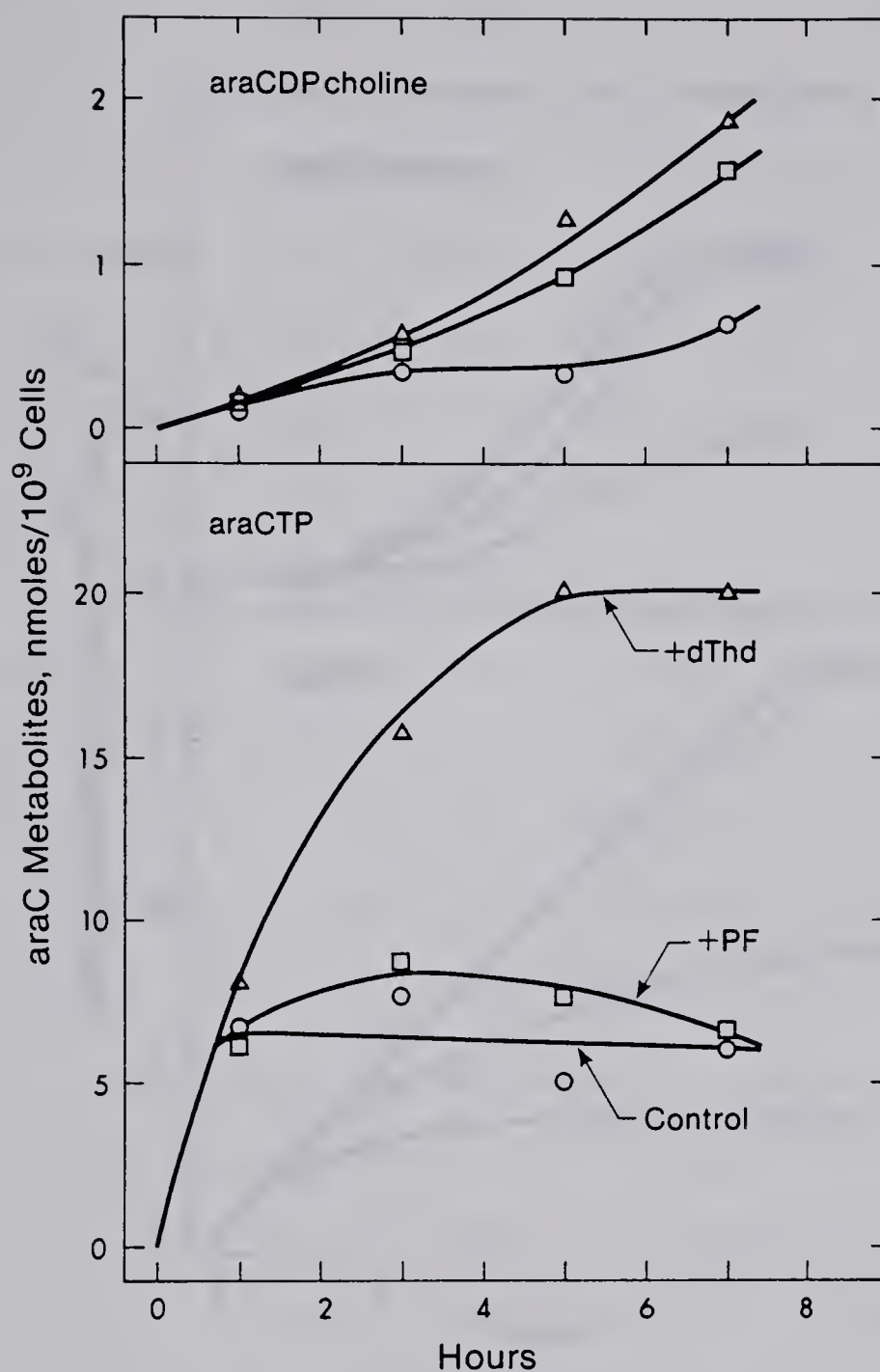


Figure 11. Influence of dThd and PF on the formation of araC metabolites in HeLa cells. Cells were cultured in medium containing 0.6 μM [5,6-³H]araC with or without 1 mM dThd or 1 μM PF. After the intervals indicated, perchloric acid extracts of the cells were chromatographed as described in Fig. 9.

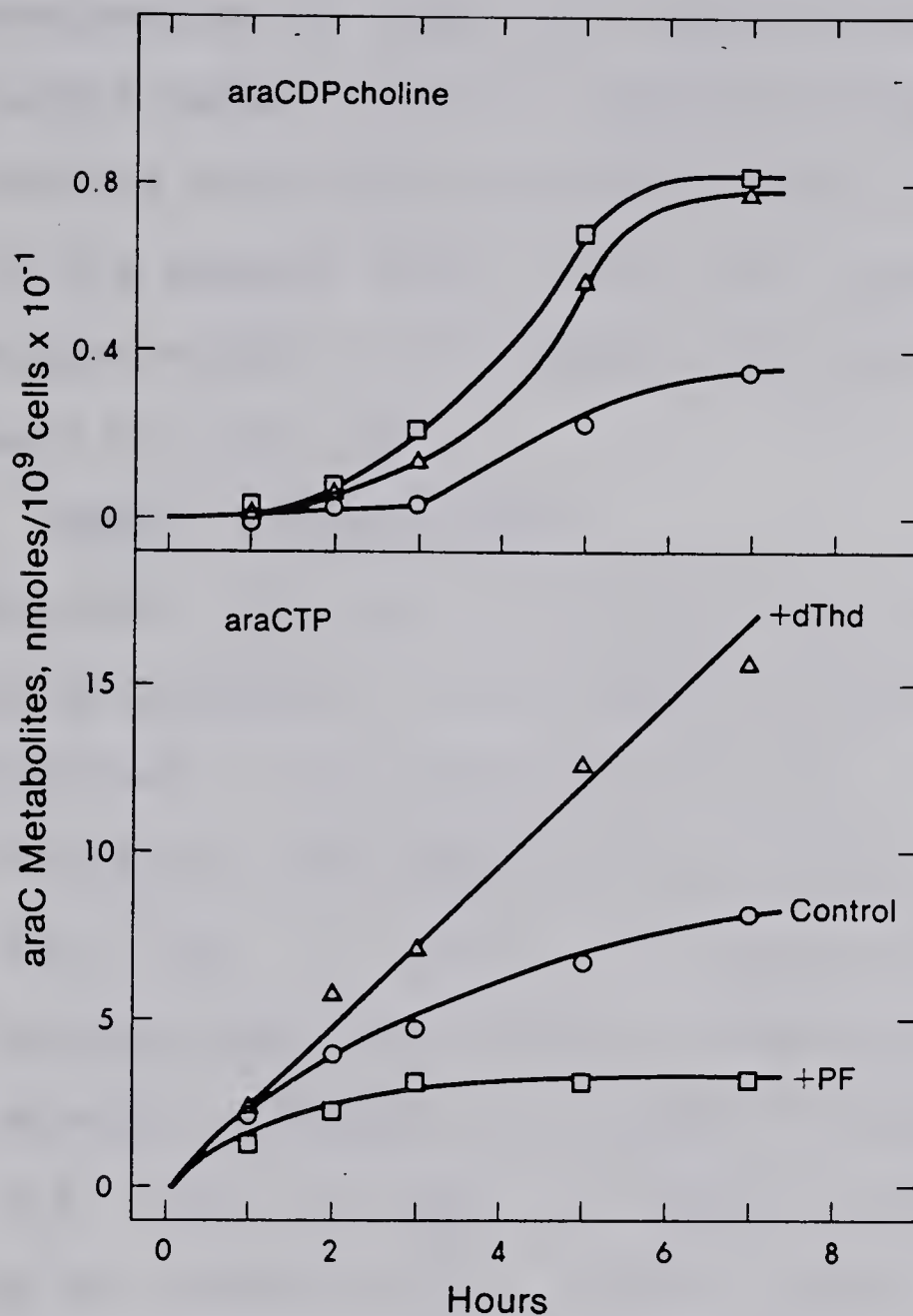


Figure 12. Effects of dThd and PF on formation of araC metabolites in L5178Y cells. Cells were cultured in Fischer's Medium containing 10% dialyzed horse serum and 3 μM [5,6- ^3H]araC (1 $\mu\text{Ci/ml}$) with or without 1 mM dThd or 1 μM PF. After the intervals indicated, perchloric acid extracts of the cells were chromatographed as described in Fig. 9.

or absence of 1 mM dThd* or 1 μ M PF. A 2.5-fold enhancement in araC anabolism is represented in the increases of the cellular concentrations of araCTP and araCDPcholine (Fig. 12) which occurred during culture in dThd-containing medium. Although PF reduced araC uptake by L5178Y cells to 76% and 48% of that in its absence after 1 and 7 hr, respectively, the araCDPcholine content of PF-treated cells was enhanced 2.2-fold after 7 hr (Fig. 12).

4. Human Leukemic Cells.

The influences of HU (400 μ M) and of dThd (1 mM) on the uptake of araC by peripheral human leukemic leukocytes in vitro was determined in the experiments of Fig. 13. Neither HU nor dThd influenced araC uptake in the leukemic cells from patient D.G. (Fig. 13, Panel A) at concentrations which consistently enhanced araC anabolism in cultured cells. Likewise, HU did not influence araC uptake by leukemic cells from patient E.B. (Fig. 13, Panel B); however, dThd inhibited araC uptake by the latter's cells, whether present alone or in combination with 10 μ M DU. The basis for this inhibitory effect of dThd is not known.

C. Discussion

This segment of our study demonstrated that dThd (1 mM) and HU (200 μ M) enhance araC anabolism in cultured RPMI 6410

*IC₅₀ values for 50 hr interval of culture in the presence of dThd was 75 μ M.

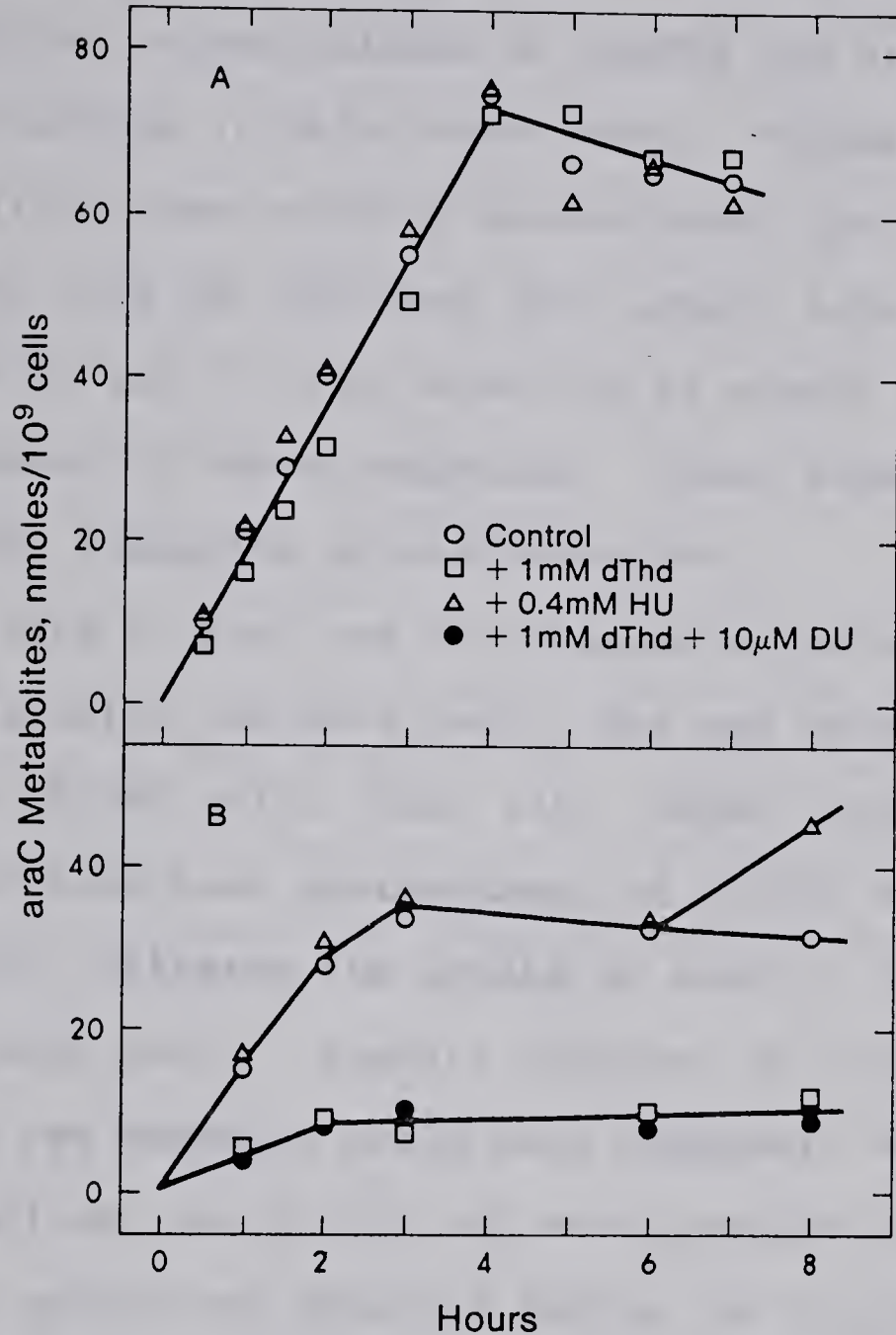


Figure 13. Uptake of araC by human leukemic cells.

Peripheral leukemic cells from patients D.G. (10-year old male with acute lymphocytic leukemia) and E.B. (38-year old female with acute myelomonocytic leukemia) were incubated in medium containing 3 μ M [5,6-³H] araC with or without 1 mM dThd, 0.4 mM HU, or 1 mM dThd and 10 μ M DU together. After the intervals indicated, total cellular radioactivity in cells from patient D.G. (Panel A) or patient E.B. (Panel B) was determined as described in Chapter II, Section D.

cells, HeLa cells and lymphoma L5178Y cells and that increases in the cellular concentrations of araCTP and araCDPcholine were major factors in this enhancement. Plagemann and coworkers (133) have recently demonstrated that (1) HU (1 mM) and dThd (100 μ M) enhanced araC uptake into Novikoff rat hepatoma cells and (2) the formation of araCTP was increased in the presence of these compounds. These workers did not assay for the formation of araCDPcholine.

The uptake of araC was not changed significantly by PF in RPMI 6410 cells and HeLa cells, but was decreased by 50% in lymphoma L5178Y cells (Fig. 12). Cadman and coworkers (20) demonstrated that pretreatment of L5178Y cells with 5 μ M PF did not influence the uptake of araC or the size of cellular araCTP pools. Results obtained by Plagemann et al. (133) using rat hepatoma cells were complex: when [5-³H]araC (0.03 μ M) and PF (20 μ M) were provided simultaneously, araC uptake was enhanced during the following 60 min; however, araC uptake was unchanged in cells pretreated for 4 hr with 20 μ M PF.

These experiments showed that PF increased the proportion of araC-derived radioactivity associated with araCDPcholine in all the cultured cell lines studied. Possibly, the depletion of cellular CTP by PF (20) precluded competition of CTP with araCTP for phosphorylcholine cytidylyltransferase. The latter authors have reported that pretreatment of L5178Y cells with PF for 1.5 to 4.5 hr (1) enhanced the subsequent toxicity of 2 hr exposures to araC, but (2) did

not influence the rate of cellular uptake of araC or the concentrations of araCTP achieved. It is interesting to speculate that enhanced formation of araCDPcholine (Fig. 12) may have contributed to enhanced araC cytotoxicity.

When peripheral leukocytes from leukemic patients were studied, neither dThd nor HU increased araC uptake at concentrations greater or equal to those which consistently enhanced araC anabolism in cultured cell lines. Reasons for the failure of DU (Chapter IV), HU and dThd to stimulate araC uptake in peripheral leukemic leukocytes were not apparent and it was felt that a better understanding of the biochemical basis of the drug-dependent enhancement of araC anabolism by cultured cells might provide further insight.

VI. Biochemical Basis of Enhancement by 3 DU of the Anabolism of AraC in RPMI 6410 Cells.

A. Introduction

The primary events comprising the cellular uptake of araC are permeation through the plasma membrane (a mediated process, Chapter IV, Section A), and phosphorylation by dCyd kinase. One aim of this work was to find criteria which would indicate whether one or both of these events were involved in the enhancement of araC anabolism resulting from the presence of DU, dThd and HU. If transport was rate-limiting in araC uptake, an increase in the number or activity of the nucleoside transport elements in the plasma membrane would be expected to enhance araC anabolism. On the other hand, if araC phosphorylation was rate limiting, an increase of the number or activity of cellular dCyd kinase molecules should stimulate araC uptake.

It has been established that dCTP is an allosteric feedback inhibitor of dCyd kinase activity (69). Hence, depletion of dCTP by DU (19) or by dThd (91,14) could explain the influence of DU and dThd on araC anabolism. However, that exerted by HU, which lowers cellular concentrations of dATP and possibly dGTP, but not those of dTTP or dCTP, remains unexplained, as does the lack of effect of PF, which depletes cellular pools of CTP and of dCTP, presumably (20). To date, allosteric modulation of the transport of araC or other nucleosides has not been demonstrated.

We have attempted to determine the basis of the DU-enhancement of araC anabolism in RPMI 6410 cells by (1)

studying the influence of culture in the presence or absence of DU on the activity of dCyd kinase in unfractionated extracts, (2) assessing the number of nucleoside transport sites on untreated and DU-treated cells using ^{35}S -labelled nucleoside transport inhibitor, and (3) determining the influence of DU treatment on the progression of cells through the cell cycle. The latter possibility was suggested by the influence of the cell cycle stage on (1) the activity of dCyd kinase (Chapter I, Section G) and (2) nucleoside transport (23). Finally, the influence of the protein synthesis inhibitor, cycloheximide (CH), on the drug-dependent enhancement of araC anabolism was studied. Some of the experiments described below also assessed the influence of culture in the presence of dThd or HU on the parameters listed above.

B. Results.

1. Deoxycytidine kinase.

Figs. 14 and 15 summarize experiments using unfractionated extracts of RPMI 6410 cells which demonstrated the validity of the assay for dCyd kinase activity. The experiments of Fig. 14 compared the following two procedures for the determination of araC phosphate formation. Method A employed selective binding of phosphorylated products in assay mixtures to DEAE cellulose paper squares (Fig. 14, Panel A) and Method B summed araCMP, araCDP and araCTP concentrations determined by TLC analysis (Fig. 14, Panel B). It is evident that the

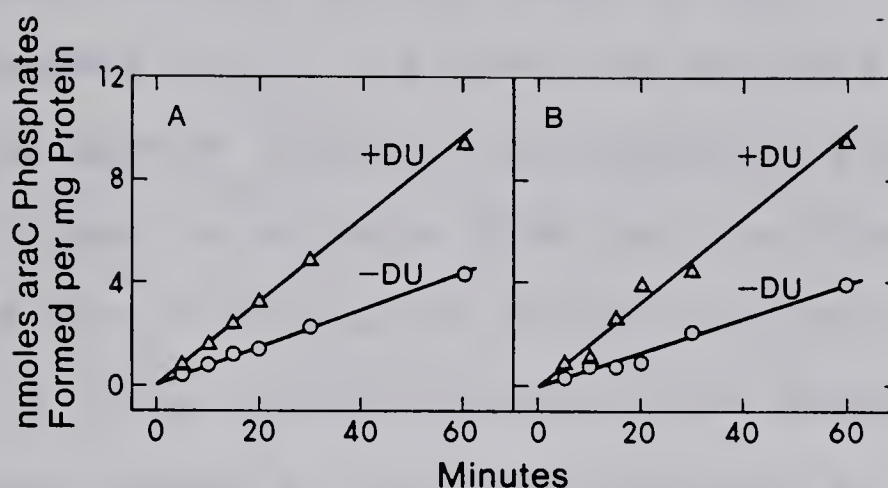


Figure 14. Formation of araC phosphates by extracts from RPMI 6410 cells. Formation of araC phosphates at 37° in incubation mixtures (Chapter II, Section H) containing 80 μ M [5,6-³H]araC (530 cpm/pmole) by cell extracts was measured at intervals by two methods. Method A: portions of the reaction mixtures were applied directly to DEAE cellulose paper squares which were rinsed and assayed for radioactivity. Method B: portions were heated (100°, 3 min), cleared by centrifugation (8000 *g*, 0.5 min) and analyzed by TLC on PEI-cellulose using solvent 1. Method B values for araC phosphates represent the sum of concentrations of the mono-, di- and triphosphates of araC. TLC analysis using solvent 3 indicated 1.8 and 9.9% conversion of araC to araU after 15 and 60 min of incubation, respectively.

former procedure (Method A) provided the more satisfactory measure of araC phosphate formation and that the rate of that process was constant for at least 60 min.

It is apparent in Fig. 14 that the activity of the araC phosphorylation system (pmoles araC phosphorylated per mg protein) was higher in extracts from cells cultured in the presence of DU for 12 hr than in extracts from untreated cells. Table 5 lists concentrations of the phosphorylated araC metabolites formed in the assay mixtures of the experiment of Fig. 14. Mono-, di- and triphosphates of araC were formed, but synthesis of araCDPcholine or of araCDPethanolamine was not evident.

Fig. 15, Panel A shows that rates of araC phosphate formation were proportional to the protein content of the assay mixtures (between 10 and 90 μ g). The stability of the dCyd kinase activity in extracts incubated at 0° is shown in Fig. 15, Panel B. The extract from cells cultured in the presence of 10 μ M DU for 12 hr lost 20% of its activity after 90 min at 0°, whereas that from control cells was stable. dCyd kinase from DU-treated or control cells was stable for at least 2 weeks at -20°.

The experiment of Fig. 16 explored the relationship between the concentration of araC and the rate of araC phosphate formation in extracts from control and DU-treated cells; reciprocal plots were linear (as found previously by Ives and Durham (69)) and indicated that both the K_m and V_{max} values of the dCyd kinase activity in extracts of DU-treated cells were greater than the corresponding parameters in

TABLE 5

Metabolites of araC formed in dCyd kinase assay mixtures

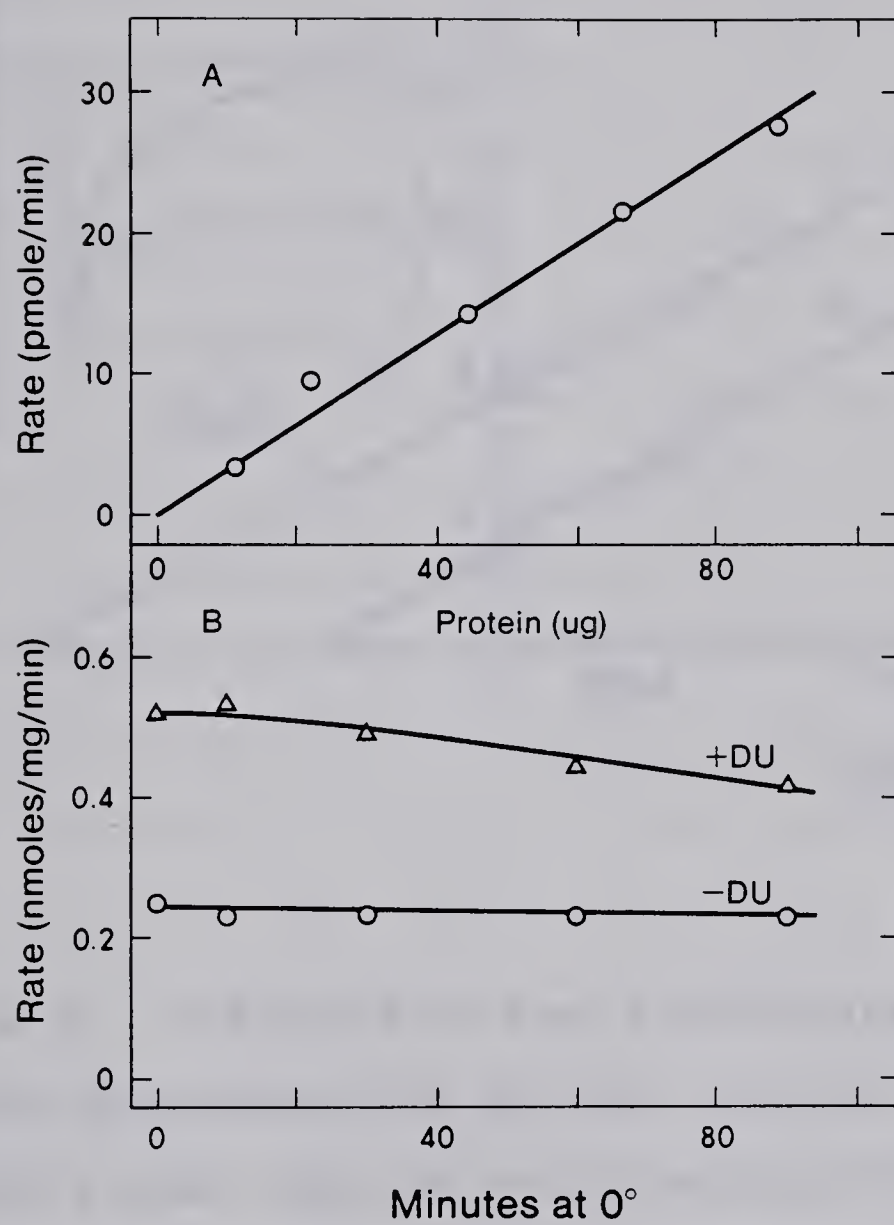
AraC phosphates formed in dCyd kinase assay mixtures were determined chromatographically as described in Fig 14, Method B.

Time (min)	DU conc (μ M) during pre- treatment ^{α}	Metabolites of araC (nmoles/mg protein)		
		araCTP	araCDP	araCMP
5	0	0.02	0.06	0.25
	10	0.14	0.17	0.63
10	0	0.21	0.02	0.55
	10	0.30	0.41	0.44
15	0	0.28	0.18	0.26
	10	0.67	0.60	1.36
20	0	0.34	0.18	0.34
	10	1.28	0.89	1.65
30	0	1.03	0.45	0.59
	10	1.29	1.09	1.82
60	0	2.61	0.62	0.72
	10	5.76	1.54	2.20

^{α} refers to drug treatment of cells prior to extraction

Figure 15, Panel A. Relationship between protein content (of dCyd kinase assay mixtures) and rate of araC phosphate formation. Assay mixtures contained 80 μ M [5,6-³H] araC (20 cpm/pmole) and graded volumes of extract from DU-treated (10 μ M, 12 hr) RPMI 6410 cells. The rate of araC phosphate formation during 30 min incubations was determined using Method A, Fig. 14.

Figure 15, Panel B. Stability of dCyd kinase at 0°. Extracts of control or DU-treated (10 μ M, 12 hr) RPMI 6410 cells were incubated at 0° for the intervals indicated, and then assayed for the ability to form araC phosphates during 15 min incubations at 37° in the presence of 80 μ M [5,6-³H]araC (27 cpm/pmole). Assay mixtures for enzyme from untreated and DU-treated cells contained 26 μ g and 43.7 μ g of cellular protein, respectively.



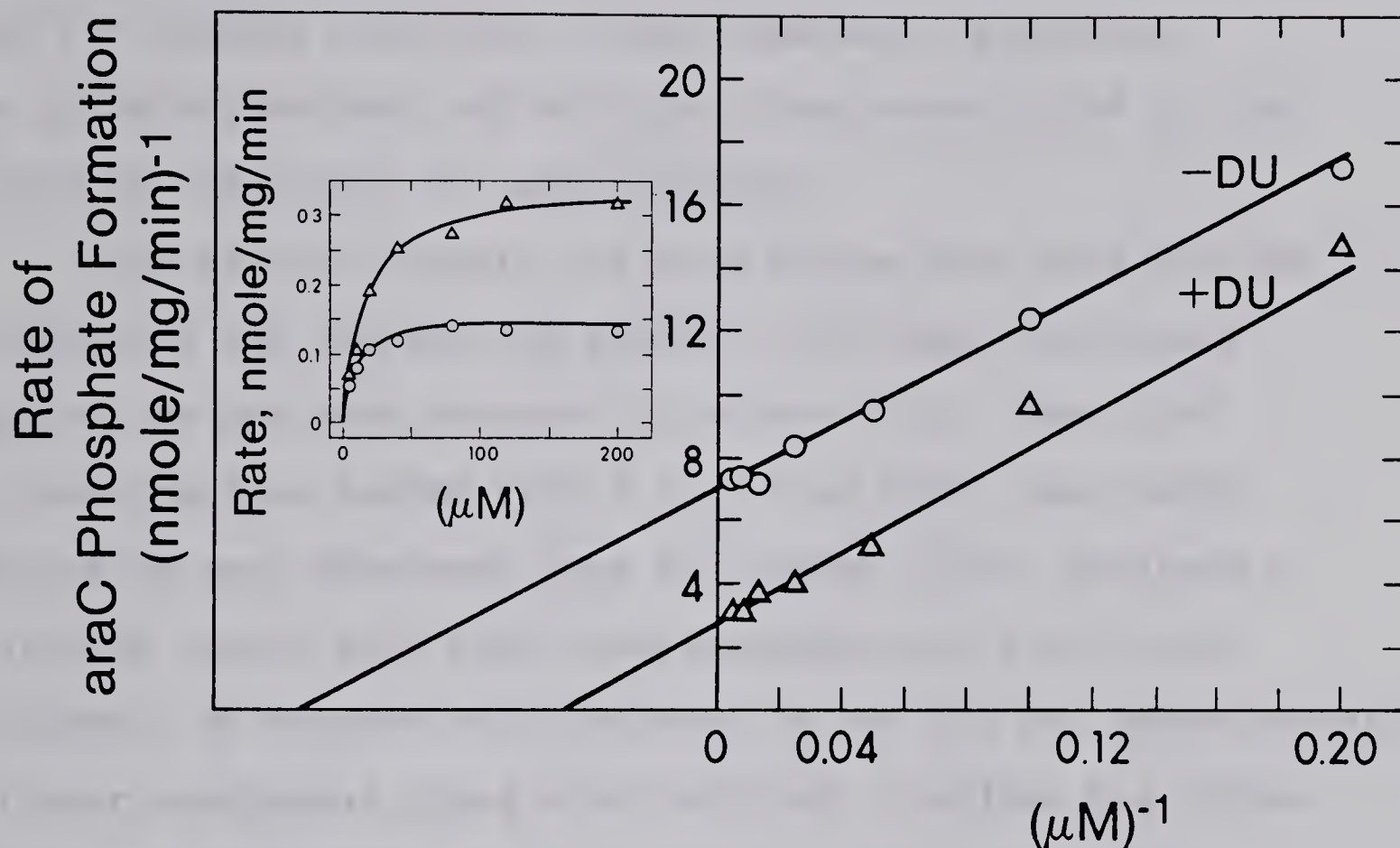


Figure 16. Influence of araC concentration on the rate of araC phosphorylation by cell extracts. The activity of dCyd kinase (rate of araC phosphate formation per mg protein) in extracts from untreated and DU-treated (10 μM , 12 hr) RPMI 6410 cells was determined during 15 min incubations at graded [5,6-³H]araC concentrations (1.72×10^5 cpm per assay mixture). Reaction volumes were 0.05 ml and contained 29.3 μg (-DU) or 25.6 μg (+DU) protein; each point represents the mean of triplicate determinations.

extracts of control cells (see Table 6, Experiment 2).

Table 6 summarizes the kinetic parameters (obtained from reciprocal plots as in Fig. 16) for dCyd kinase activity found in cells cultured in the presence or absence of DU or HU for varying intervals. Each experiment represents a different extract and straight lines were fitted to the data by the method of least squares.

The kinetic behavior of dCyd kinase when dCyd was the substrate was complex and yielded non-linear reciprocal plots, as has been reported by others (69). When dCyd concentrations ranged from 0.5 to 5 μM dCyd, zero order kinetics were obtained; Ives and Durham (1969) obtained a similar result with dCyd concentrations of 10 to 50 μM . However, at concentrations between 10 and 120 μM , approximately linear reciprocal plots were obtained, yielding the values reported for Experiment 1 in Table 6 (for a graphical illustration, see Fig. 23). K_m and V_{\max} values for araC phosphorylation by crude extracts from untreated cells were 2- to 4-fold greater than corresponding parameters for dCyd phosphorylation. When dCyd (at concentrations greater than 20 μM) was substrate, the V_{\max} of dCyd kinase activity in extracts from DU-treated cells was increased 3.2-fold relative to that from untreated cells, but the K_m was unchanged (Table 6, Experiment 1). When araC was substrate, both K_m and V_{\max} values for dCyd kinase activity were enhanced in extracts from DU-treated cells (Table 6).

TABLE 6

Kinetic parameters of dCyd kinase in extracts from RPMI 6410 cells

The dCyd kinase activity of extracts prepared from control or drug-treated RPMI 6410 cells was determined at graded substrate (dCyd or araC) concentrations as described in Fig. 16.

Experiment No.	Substrate	Treatment ^a		Kinetic Parameters	
		Drug	Interval (hr)	K (μ M)	V _{max} (nmole/mg protein/min)
1	dCyd	none		3.40	0.06
		DU	12	3.73	0.18
2	araC	none ^b		7.54	0.14
		DU ^b	12	22.73	0.37
		none ^c		8.78	0.22
		DU ^c	12	18.38	0.47
3	araC	none		6.08	0.21
		DU	12	19.28	0.46
4	araC	none		6.18	0.24
5	araC	none		7.26	0.20
		DU	12	15.55	0.53
6	araC	none		7.55	0.16
		DU	12	20.22	0.45
7	araC	none		6.53	0.16
		HU	4	10.24	0.27
		HU	12	23.84	0.50
8	araC	none		6.47	0.17
		HU	4	11.17	0.27

^aPrior to preparation of extracts, cells were cultured in the presence of 10 μ M DU or 200 μ M HU for the intervals specified.

^bExperiment of Fig. 16.

^c1 ml of cell extract was dialyzed for 8 hr against 50 ml of extraction buffer at 4° with 3 changes of buffer.

DU (10 μ M) added to mixtures assaying activity from control cells did not influence the activity of dCyd kinase.

When extracts from DU-treated or untreated cells were dialyzed against extraction buffer, both K_m and V_{max} values for activity of enzyme from DU-treated cells remained elevated relative to values from untreated cells when araC was substrate (Table 6, Experiment 2). Furthermore, when heated extract (100°, 3 min) from DU-treated cells was added to control assay mixtures, K_m and V_{max} values for araC phosphorylating activity were not altered. When extracts from untreated and DU-treated cells were present together in the same assay mixtures, the resulting activity, in terms of araC phosphates formed, was approximately the sum of the activities in the individual extracts (Table 7). Together, these observations demonstrate that the enhanced activity of dCyd kinase in extracts from DU-treated cells is not attributable to (1) the presence of dissociable activators, (2) the presence of feedback inhibitors (e.g. dCTP) in extracts from control cells, or (3) a direct influence of DU or its anabolites present in extracts from DU-treated cells.

Culture of RPMI 6410 cells with 200 μ M HU prior to preparation of cell extracts also elevated V_{max} and K_m values of the dCyd kinase activity (when araC was substrate) in a time-dependent fashion: K_m values of the activity from cells cultured in the presence of HU for 4 and 12 hr were 1.7- and 3.6-fold greater than those of enzyme from untreated cells, respectively (Table 6, Experiments 7 and 8). V_{max}

TABLE 7

dCyd kinase activity in mixtures of extracts from
untreated and DU-treated RPMI 6410 cells

The activity of dCyd kinase in extracts prepared from cells cultured in the presence or absence of 10 μ M DU for 16 hr was measured during 30 min incubations; the extracts from control and DU-treated cells contained 12.8 and 16.8 μ g cellular protein per 5 μ l, respectively. Results represent means \pm S.D. of triplicate determinations.

Extract	pMoles araC phosphates formed per min
1. 5 μ l from control cells	3.64 \pm 0.34
2. 5 μ l from DU-treated cells	7.07 \pm 0.32
3. 5 μ l from control cells + 5 μ l from DU-treated cells	10.04 \pm 0.33
4. Expected activity if 1 and and 2 were additive	10.71

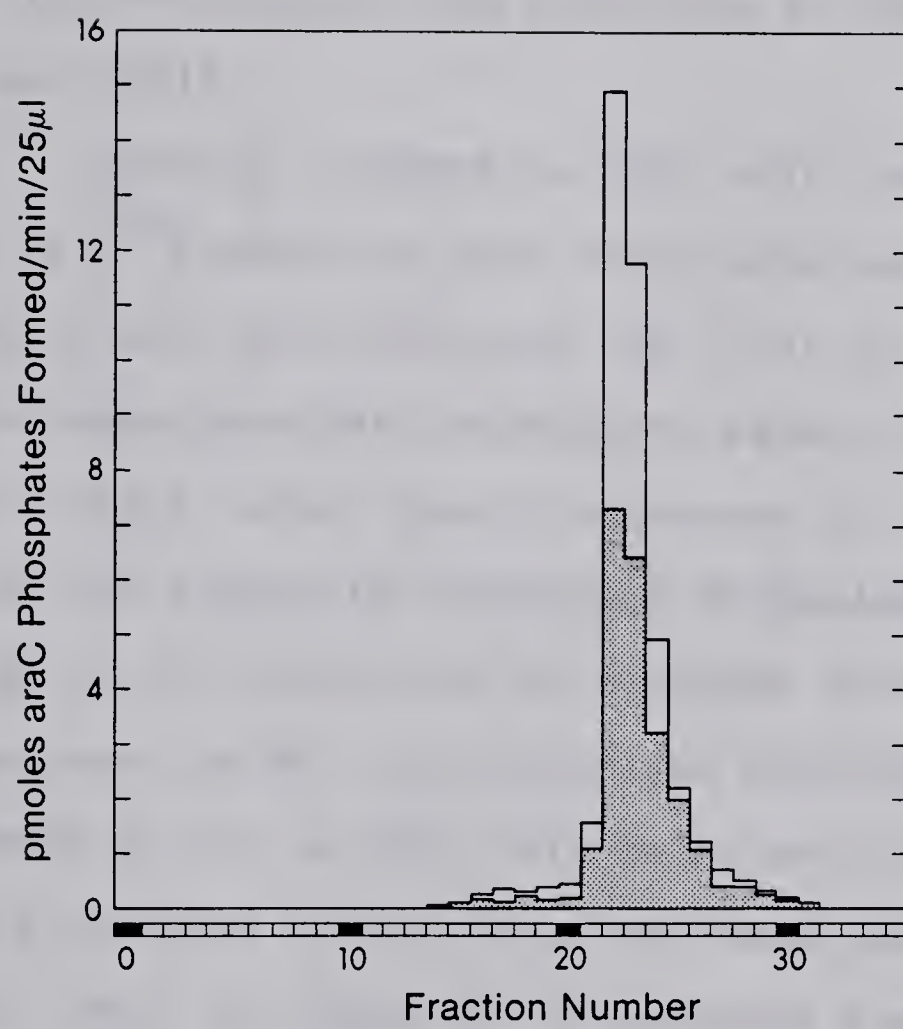
values were enhanced similarly. The provision of 200 μ M HU in assay mixtures did not influence dCyd kinase activity.

dCyd kinase in unfractionated extracts (containing 4.2 mg protein) from DU-treated or untreated RPMI 6410 cells were partly purified by affinity chromatography on columns of Sepharose 4B containing covalently bound dThd (Chapter II, Section H). Elution was accomplished as described in Fig. 17; fractions 22 to 25 (inclusive) contained most of the araC phosphorylating activity in both enzyme preparations. Extract from DU-treated cells contained 1.8-fold more araC-phosphorylating activity in these fractions than did extract from untreated cells containing an equivalent amount of protein. No other peaks of araC-phosphorylating activity eluted from these columns, even when eluant was 0.8 M Tris-HCl buffer (pH 7.5). Cheng et al. (28) reported that leukemia cells (human AML) contained cytoplasmic and mitochondrial isozymes of dCyd kinase which were separable on dThd-containing Sepharose. However, araC did not serve as a substrate for the mitochondrial isozyme; therefore, the single peaks of activity observed in the experiment of Fig. 17 probably represented the cytoplasmic isozyme of dCyd kinase. These results suggest that cells cultured in the presence of DU for 12 hr contained either (1) more molecules of dCyd kinase than untreated cells, (2) modified molecules of dCyd kinase with greater catalytic activity, or (3) both (1) and (2).

The activity of dCyd kinase was also enhanced in extracts prepared from HeLa cells cultured for 6 hr in the

Figure 17. Affinity chromatography of dCyd kinase.

Extracts (1 ml, 4.2 mg protein) from untreated (shaded portion) and DU-treated (10 μ M, 12 hr) RPMI 6410 cells were applied to 0.5 x 2 cm columns of dThd-containing Sepharose 4B gel previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 10% glycerol and 5 mM dithioerythritol. Elution (1 ml fractions) was accomplished at 4° with the following solutions containing glycerol and dithioerythritol as above: fractions 2 to 11, equilibration buffer; fractions 12 to 17, 0.2 M Tris-HCl buffer (pH 7.5); fractions 18 to 20, the latter containing 100 μ M dThd; fractions 21 to 23, 0.4 M Tris-HCl buffer (pH 7.5); fractions 24 to 28, the latter containing 300 μ M dThd and fractions 29 to 33, 0.4 M Tris-HCl buffer (pH 7.5) containing 600 μ M dThd. Immediately after collection, ovalbumin (0.2 mg) was added to each fraction; 25 μ l samples of each fraction were then assayed for dCyd kinase activity. Reaction volumes were 100 μ l and contained 40 μ M [5,6-³H]araC as substrate. A confirmatory experiment yielded similar results.



presence of either 200 μ M HU or 1 mM dThd. Kinase activities (nmoles araC phosphates formed/mg protein/min) determined in the presence of 10 and 40 μ M [5,6-³H]araC, respectively, were as follows: untreated cells, 0.014 and 0.016; HU-treated cells, 0.018 and 0.031; dThd-treated cells, 0.018 and 0.030. Corresponding values for activity in extracts from HeLa cells treated for 6 hr with 10 μ M DU (a concentration which did not enhance araC anabolism in these cells) were 0.012 and 0.016.

2. Binding of NBMPA to RPMI 6410 Cells.

The binding of [³⁵S]NBMPA to RPMI 6410 cells was compared in untreated cells and cells cultured for 12 hr in the presence of DU. This comparison was intended to determine whether NBMPA binding might detect possible changes in the number or properties of the nucleoside transport mechanism of RPMI 6410 cells under the conditions of enhanced araC anabolism following exposure to DU. Site-specific binding (as defined below) of NBMPA at 37° to RPMI 6410 cells was complete within 15 min, whether or not cells had been pretreated with DU (Fig. 18). Fig. 19 (Panel B) illustrates the basis of the binding assay. The binding of NBMPA was greatly reduced in the presence of its congener, NBMPR, over a wide range of concentrations. Site-specific binding of [³⁵S]NBMPA, defined as the difference between the cellular content of ³⁵S in the presence or absence of NBMPR, is shown in Fig. 19 (Panel A) to be a saturable function of NBMPA concentration. It may be noted that the [³⁵S]NBMPA content of medium and

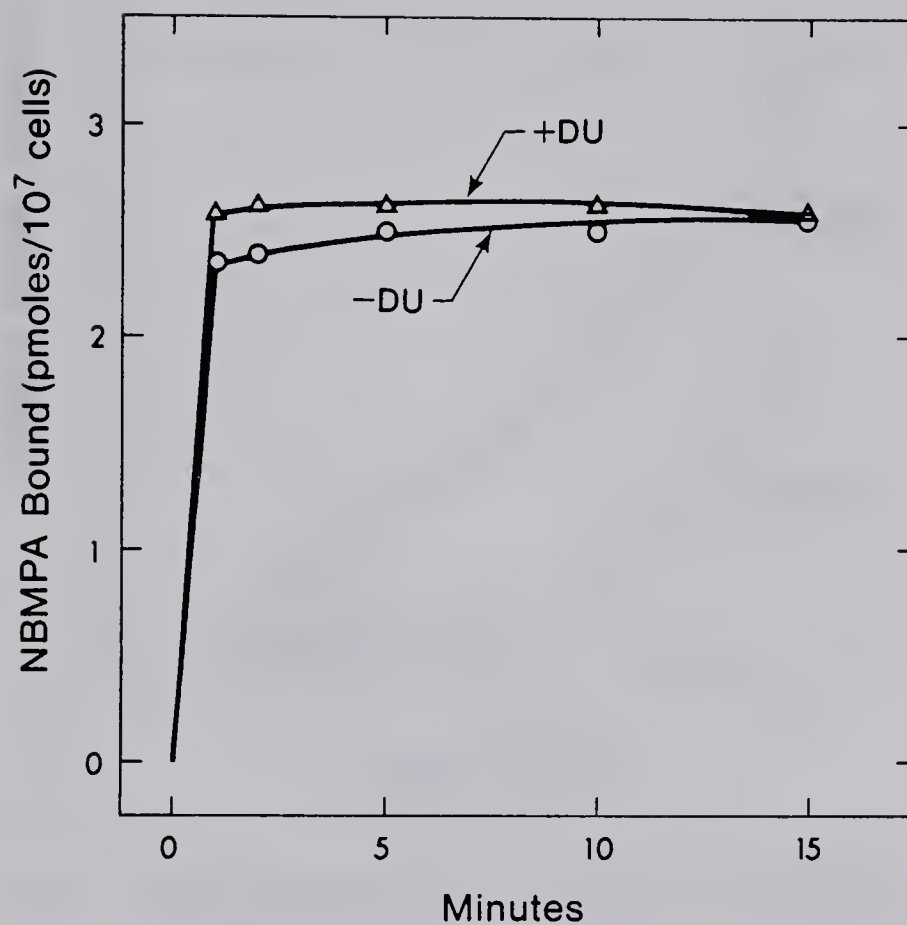


Figure 18. Binding of NBMPA by RPMI 6410 cells. Cells cultured in the presence or absence of 10 μ M DU for 12 hr were resuspended in RPMI 1640 medium (minus serum) with or without 5 μ M NBMPR. After 5 min at 37 $^{\circ}$, medium containing [35 S]NBMPA (final concentration, 2.8 nM) was added and, after the intervals indicated at 37 $^{\circ}$, site-specific binding of NBMPA was determined as described in Fig. 19.

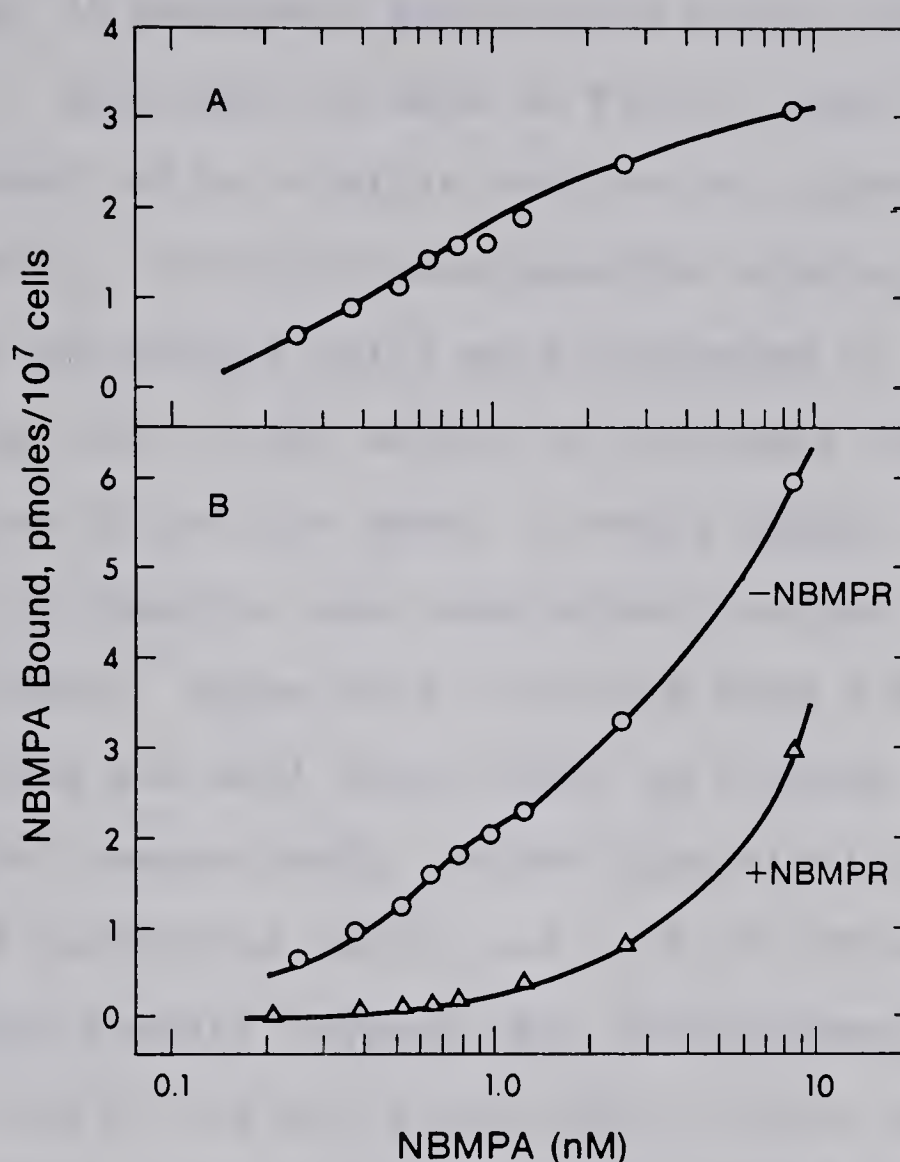


Figure 19. Site-specific binding of NBMPA by RPMI 6410 cells; relationship between ligand concentration and binding. Cells cultured in the presence or absence of 10 μ M DU for 12 hr were resuspended in RPMI 1640 medium with or without 5 μ M NBMPR; after 5 min at 37 $^{\circ}$, medium containing [35]NBMPA (912 cpm/pmole) was added to each incubation mixture, achieving a range of final concentrations. After 15 min at 37 $^{\circ}$, the 35 S-content of the cells in each incubation mixture was measured in duplicate by a centrifugation method (see Appendix A) and is shown in Panel B. The site-specific binding of NBMPA was determined as the difference between binding in the presence and absence of NBMPR (Panel A).

cells in Fig. 19 represent equilibrium conditions (85; see Appendix A). Although the data of Fig. 19 were obtained with DU-treated cells, similar results were observed with untreated cells. Data for site-specific binding of NBMPA to untreated or DU-treated cells were subjected to mass law analysis (Fig. 20) by the method of Scatchard (49): since straight lines fitted the data, it would appear that a single type of receptor was responsible for the site-specific binding of NBMPA. These data indicated that 2.0×10^5 and 2.1×10^5 sites per cell bound NBMPA in control and DU-treated cells, respectively. NBMPA dissociation constants were 1.01 nM (untreated cells) and 1.03 nM (DU-treated cells). These results suggest that pretreatment of RPMI 6410 cells with DU did not significantly alter the characteristics of NBMPA binding to these cells; a confirmatory experiment yielded similar results.

3. Cell Cycle Arrest of RPMI 6410 Cells by DU.

In this section, consideration has been given to the idea that the DU-induced enhancement of araC uptake and dCyd kinase activity in RPMI 6410 cells might be due in part to arrest by DU of cell cycle progression. Fig. 21 illustrates the influence of exposure to DU upon the frequency distribution of the DNA content in RPMI 6410 cell populations. As the exposure interval increased, the proportion of cells in the $G_2 + M$ phases of the cell cycle decreased, while the G_1 peak of the distribution broadened and shifted towards a higher DNA content. These results suggested that, following

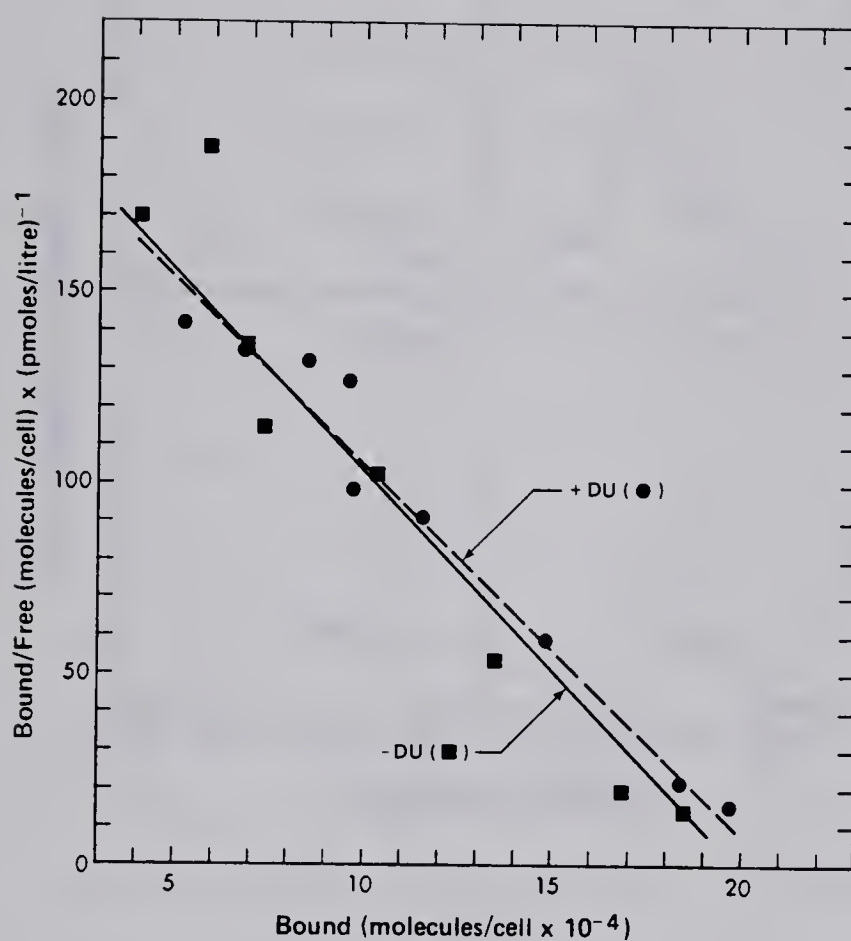


Figure 20. Mass law analysis of NBMPA binding data from Fig. 19. Data from Fig. 19 for site-specific binding of [³⁵S]NBMPA to RPMI 6410 cells cultured in the presence or absence of 10 μ M DU for 12 hr are presented here in the form of mass law (Schatchard) plots. Lines were fitted to the data by the method of least squares.

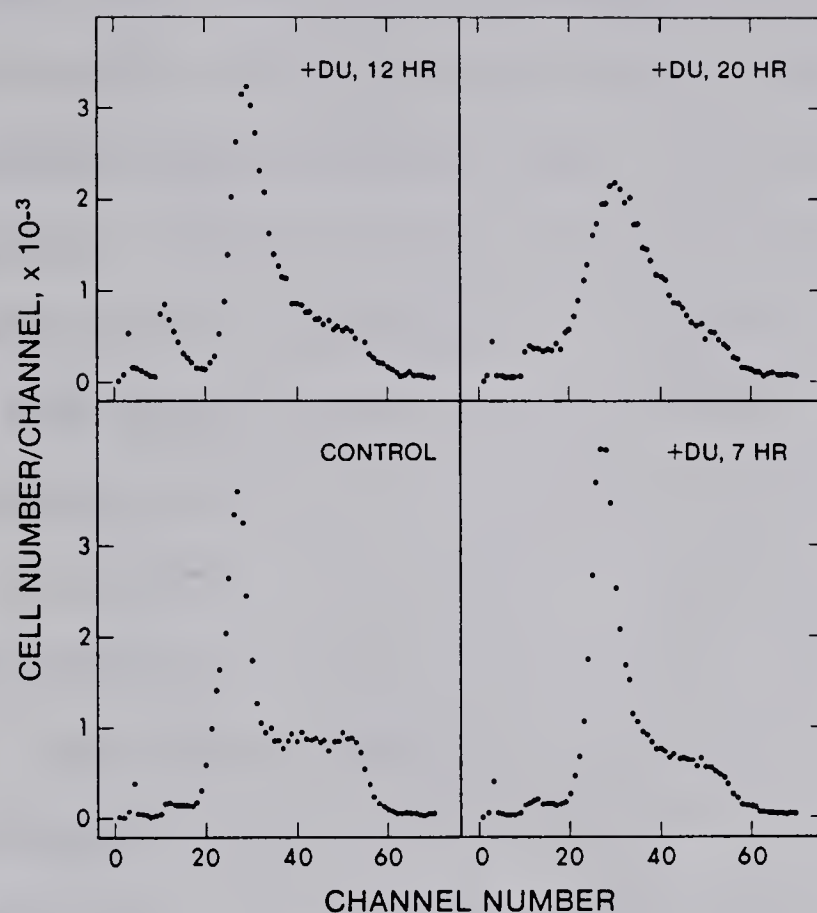


Figure 21. Distribution of the DNA content in RPMI 6410 cells. After exposure to 10 μ M DU for the times indicated, the frequency distribution of cellular DNA content was determined by flow microfluorometry. A confirmatory experiment yielded similar results.

DU exposure for 16 hr, cells accumulated at a stage in S phase at which their DNA content was slightly greater than $2n$.

When RPMI 6410 cells were incubated for 16 hr in medium containing $10\ \mu\text{M}$ DU, DNA synthesis ceased, but upon transfer to DU-free medium supplemented with $2\ \mu\text{M}$ Cyd, DNA synthesis resumed rapidly. Incorporation of $[5,6\text{-}^3\text{H}]\text{dThd}$ into acid-insoluble material was employed as a measure of DNA synthesis. The rate of DNA synthesis was maximal about 2 hr after the change of medium and then decreased to a plateau level by 8-10 hr (Fig. 22, Panel A); a second broader peak of DNA synthesizing activity occurred between 16 and 25 hr (data not shown). Following transfer to DU-free medium without Cyd, DNA synthesis began after a 1-2 hr interval which, perhaps, represents the interval necessary for replenishment of cellular cytidine and deoxycytidine phosphates. Table 8 demonstrates that, at concentrations between 2 and $10\ \mu\text{M}$, Cyd was more effective than dCyd in restoring DNA synthesizing activity in RPMI 6410 cells following exposure to DU for 16 hr. It is noted that $2\ \mu\text{M}$ dCyd was more effective than $5\ \mu\text{M}$ dCyd in the restoration of DNA synthesis; it is possible that this difference represents impairment of dThd anabolism by dCyd or a metabolite thereof.

Fig. 22 (Panel B) shows that restoration of DNA synthesis in DU-treated cells was followed by cell division, as indicated by increases in cell concentration and corresponding changes in mitotic index; in 3 experiments, cell concentra-

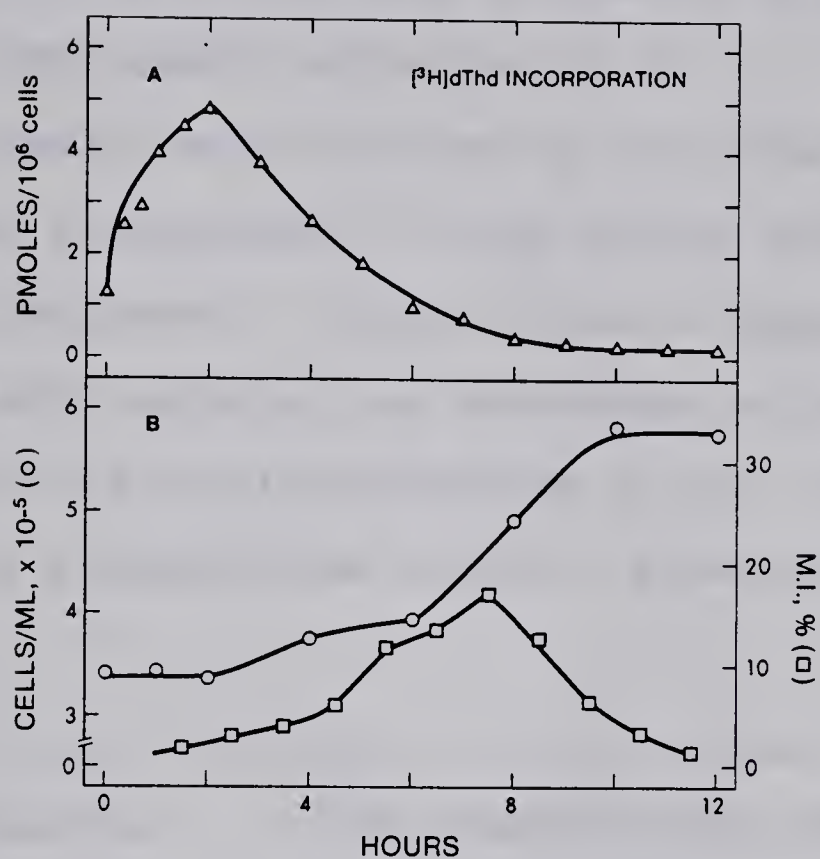


Figure 22. Synchronization of RPMI 6410 cells by DU. After exposure to 10 μ M DU for 16 hr, cells were resuspended in growth medium supplemented with 2 μ M Cyd and incubated at 37^o under culture conditions. Measured at the intervals indicated were incorporation of dThd into acid-insoluble material (Panel A), and mitotic index (M.I.) and cell concentration (Panel B). Two confirmatory experiments conducted collaboratively with Dr. Suh-er Yang of this laboratory yielded similar results.

TABLE 8

Effects of Cyd and dCyd on the incorporation of dThd into acid-insoluble material following release of RPMI 6410 cells from growth inhibition by DU

RPMI 6410 cells were incubated in the presence of 10 μ M DU for 16 hr and resuspended in fresh medium with or without nucleosides as indicated. Incorporation of [methyl- 3 H]dThd into acid-insoluble material was determined on duplicate samples after 10 and 75 min incubation at 37°. This experiment was performed in collaboration with Dr. Suh-er Yang of this Laboratory.

Concentration (μ M)		dThd Incorporation (pmoles/ 10^6 cells)	
dCyd	Cyd	10 min	75 min
0	0	0.66	0.76
2	0	1.54	5.01
5	0	1.55	2.59
10	0	1.48	2.39
0	2	2.15	8.03
0	5	3.50	8.03
0	10	4.18	8.32
5	5	1.97	5.30

tions had increased by 70-80% by 12 hr after the medium change. The shoulder on the cell concentration profile (Fig. 22, Panel B), also evident in the mitotic index data, was apparent in 2 similar experiments. This result suggests that in the presence of DU, cells were arrested at 2 loci in the cell cycle. The immediate initiation of DNA synthesis following transfer of DU-treated cells to Cyd-containing medium indicates that most of the arrested cells were in S phase; the earlier, minor increase in cell numbers suggests that cell cycle progression of a small portion of the cell population may have been blocked at the S-G₂ border.

Activities of dThd kinase change markedly during the cell replication cycle (see Chapter I, Section G). When RPMI 6410 cells were incubated for 12 hr in the presence of 10 μ M DU, the specific activities of dThd kinase (Fig. 23, Panel A) and dCyd kinase (Fig. 23, Panel B) in cell extracts were elevated relative to those from cells incubated without DU. These data suggest that in the presence of DU, cells were arrested at a stage in S phase at which the kinase activities are elevated.

Table 9 lists specific activities of dCyd and dThd kinases at various intervals after resuspension of DU-treated cells in DU-free medium supplemented with 2 μ M Cyd. Both activities peaked 1 to 2 hr after resuspension, declined gradually thereafter and were lowest immediately after mitosis. These data indicate that the peaks of dCyd and dThd kinase activities coincided approximately with the

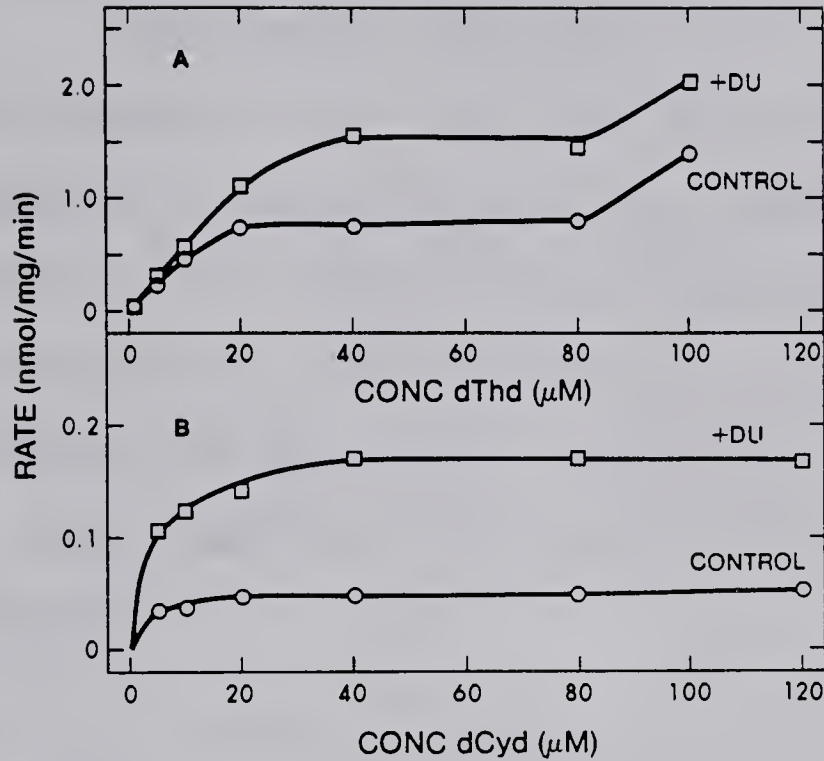


Figure 23. dThd and dCyd kinase activity in RPMI 6410 cell extracts. Following incubation of RPMI 6410 cells with or without 10 μ M DU for 12 hr, cell extracts were assayed for dThd or dCyd kinase activity. dThd kinase assay mixtures contained 25.2 μ g (control) and 27.3 μ g (+DU) of cellular protein; dCyd kinase assay mixtures contained 18.1 μ g (control) and 18.5 μ g (+DU) of protein. Rates are expressed as nmoles of dThd or dCyd phosphates formed per mg protein per min.

TABLE 9

Specific activities of dCyd kinase and dThd kinase in extracts prepared from RPMI 6410 cells at intervals following release from growth inhibition by DU

After exposure to 10 μ M DU for 16 hr, RPMI 6410 cells were resuspended in growth medium supplemented with 2 μ M Cyd and incubated at 37° under culture conditions. After the intervals indicated, cell extracts were prepared and assayed for dCyd kinase and dThd kinase activities, as previously described; assay mixtures contained 30 μ M [5-³H]dCyd or [methyl-³H]dThd.

Time (hr) after resuspension in DU-free medium	Rate (nmole/mg protein/min) ^α	
	<u>dThd kinase</u>	<u>dCyd kinase</u>
0	0.59	0.03
1	0.87	0.03
2	0.83	0.02
4	0.61	0.02
6	0.59	0.02
8	0.52	0.01
10	0.37	0.01
12	0.22	0.01

^αCorresponding activities for enzyme prepared from exponentially proliferating, untreated RPMI 6410 cells were: dThd kinase, 0.358 nmole/mg protein/min; dCyd kinase, 0.020 nmole/mg protein/min.

peak of DNA synthesis (Fig. 22, Panel A). Reports by others have shown that the cell cycle profile of dCyd kinase activity correlates with DNA synthesis; however, with some cell types (see Chapter I, Section G) dThd kinase activity appears highest after the peak of DNA synthesis, that is, in late S-phase and G₂-phase.

4. Effect of Cycloheximide on the Drug-Enhanced Anabolism of AraC.

The effect of 0.3 mM CH on the enhancement of araC uptake by DU, HU and dThd in RPMI 6410 cells is illustrated in Fig.

24. At this concentration, CH reduced the incorporation of [4,5-³H]leucine (0.07 μ M, 4 μ Ci/ml) into the acid-insoluble fraction of RPMI 6410 cells to less than 1% of that in its absence during 60 min of incubation. It is evident from Fig. 24 (Panel A) that CH alone did not alter the uptake of araC by RPMI 6410 cells during 12 hr of culture. CH did not influence the initial enhancement of araC anabolism by HU, but increases in araC uptake did not occur after 6 hr (Fig. 24, Panel A). Increases in araC uptake by DU and dThd were similarly affected (Fig. 24, Panels B and C). Furthermore, when RPMI 6410 cells cultured in the presence of 200 μ M HU and 3 μ M [5,6-³H]araC for 5 hr were then incubated in the same concentrations of HU and [5,6-³H]araC plus 0.3 mM CH, the latter did not influence the HU-dependent enhancement of araC anabolism for a 6 hr interval. Hence, the late inhibition of HU effects by CH in Fig. 24 (Panel A) must have been a consequence of the prolonged presence of CH and not of

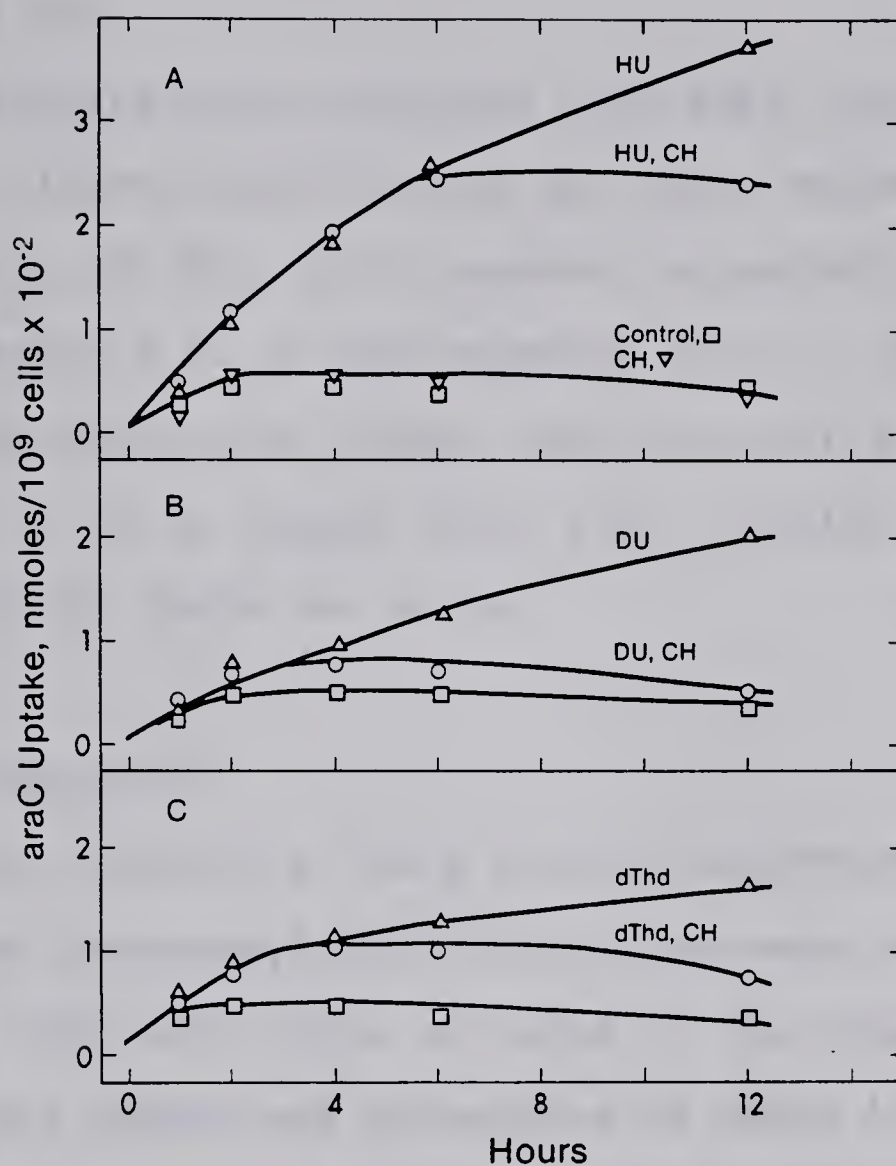


Figure 24. Effects of HU, DU and dThd on araC uptake by RPMI 6410 cells in the presence or absence of CH. Cells were cultured in the presence of 3 μM [5,6-³H]araC without (control) or with 200 μM HU (Panel A), 10 μM DU (Panel B) or 1 mM dThd (Panel C) for the intervals indicated. CH was included in some cultures to evaluate its effect on araC uptake in control cultures (Panel A) and that stimulated by HU (Panel A), DU (Panel B) or dThd (Panel C). Total cellular radioactivity was determined as described in Chapter II, Section D. Two confirmatory experiments yielded similar results.

specific CH-sensitive events taking place 6 hr after the provision of HU.

These results were confirmed with HeLa cells; CH (0.3 mM) did not affect significantly the early enhancement of araC uptake by HU (Fig. 25); however, stimulatory effects of HU decayed after 2 hr in the presence of CH. In the absence of HU, CH inhibited araC uptake only marginally after culture intervals of 4 hr or longer (Fig. 25). Similar results were observed with DU (data not shown).

C. Discussion.

The data reported in this section demonstrate that (1) dCyd and araC phosphorylating activities were enhanced in extracts of RPMI 6410 cells cultured in the presence of DU or HU, (2) the number and properties of NBMPA binding sites (presumed to be a measure of the number of nucleoside transporters) in RPMI 6410 cells were unchanged by DU-treatment, (3) DU-treatment accumulated RPMI 6410 cells at a locus within S phase of the cell cycle and (4) prolonged culture in the presence of CH prevented the enhancement of araC anabolism by DU, HU or dThd.

On the basis of these observations and of results obtained by others, the following mechanisms are proposed to explain the DU-dependent enhancement of araC anabolism in RPMI 6410 cells. When these cells were cultured in the presence of 3 μ M araC, cellular concentrations of araC nucleotides became constant within 2 hr (Table 3 and Fig. 24; ref 97), indicating that rates of araC anabolism and

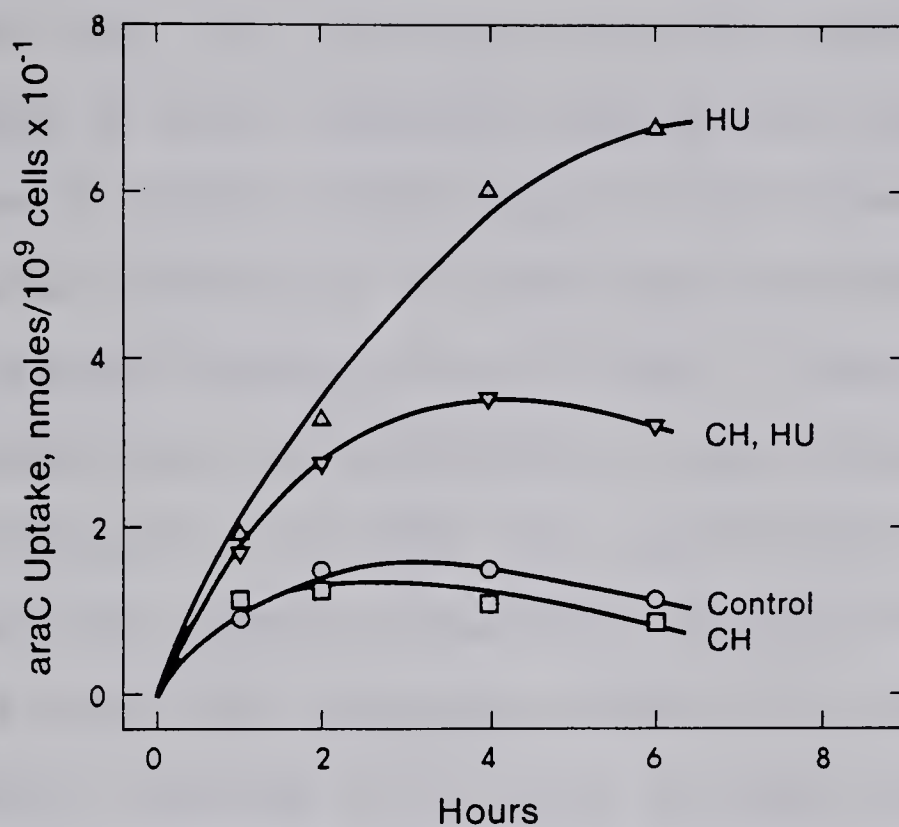


Figure 25. Effect of CH on HU-enhanced araC uptake in HeLa cells. Cells in monolayer culture were incubated in medium containing 0.6 μM [5,6-³H]araC without (control) or with 200 μM HU, 0.3 mM CH or both of the latter. After the intervals indicated, total cellular radioactivity was determined in 3 monolayers.

catabolism had become equal. However, when 9 μ M DU was present, concentrations of araC nucleotides expanded for at least 12 hr (Fig. 24); the rate of araC anabolism was thus greater than that of araC catabolism throughout that interval, a consequence of either stimulation of araC anabolism or inhibition of catabolism (of araC and araC metabolites) by a phosphorylated metabolite of DU (97). Since the depletion of Cyd phosphates, and presumably of dCyd phosphates, was complete within 6 hr in RPMI 6410 cells cultured with 9 μ M DU (Appendix B), effects resulting directly from dCTP depletion would have been fully expressed within 6 hr after provision of DU. Thus, although the release of dCyd kinase from feedback inhibition by dCTP (69) may contribute to the enhancement of araC phosphate formation by DU, other mechanisms must be involved.

DU blocked the cell cycle progression of RPMI 6410 cells at a point in S phase where the cellular activities of dCyd and dThd kinases were enhanced. Since the population doubling time of RPMI 6410 cells is 17 to 19 hr, DU-dependent accumulation of these cells in S phase could account for the 12 hr interval of expansion of araC nucleotide concentrations when DU was present. The following observations suggest that, as a result of S phase blockade, cells cultured in the presence of DU contained a greater number of dCyd kinase molecules: (1) crude extracts of DU-treated (12 hr) cells contained more araC and dCyd phosphorylating activity per mg protein than did extracts from untreated cells, (2) the en-

hancement of dCyd kinase activity in extracts from DU-treated cells was not a consequence of the presence of activators nor of the absence of inhibitors, (3) the dCyd kinase activities from untreated and DU-treated cells behaved similarly in chromatography on dThd Sepharose, and (4) when subjected to affinity chromatography, extracts from DU-treated cells yielded more araC phosphorylating activity than did similar amounts (protein) of extract from untreated cells. We suggest that the accumulation of S phase cells with enhanced levels of dCyd kinase and depleted concentrations of the latter's allosteric inhibitor, dCTP, may account for the stimulation by DU of araC nucleotide formation in RPMI 6410 cells. The prevention of the DU-dependent expansion of araC phosphate concentrations during prolonged incubations with CH (Fig. 24) can be explained in two ways: either (1) the synthesis of new dCyd kinase molecules was responsible, at least in part, for the enhancement by DU of araC anabolism, or (2) cycloheximide blocked cell cycle progression at a locus in G_1 phase [as was suggested by others: Highfield and Dewey (63) and Terasima and Yasukawa (158)] such that only a fraction of the cells (those occupying the interval between the blockade by CH in G_1 phase and that by DU in S phase) incubated with DU were accumulated at a cell cycle position corresponding to elevated dCyd kinase activity. Experiments failed to demonstrate an effect of DU treatment on the number or properties of NBMPA binding sites in RPMI 6410 cells.

Inhibition by DU of araC-araC metabolite catabolism could contribute to the enhancement of cellular araC phosphate concentrations. At this point in the present study, it was not apparent whether DU influenced araC catabolism in RPMI 6410 or other types of cells; however, results presented in Chapter VII suggest that neither DU nor its metabolites influence araC catabolism.

The properties of dCyd kinase in extracts from cells synchronized in S phase by DU treatment were intriguing in that K_m and V_{max} values for araC phosphorylating activity in these extracts were enhanced relative to corresponding parameters in extracts from untreated cells (Table 6). In contrast K_m values for dCyd phosphorylating activity (for concentrations of dCyd greater than 10 μ M) were similar in extracts of DU-treated and untreated cells, while V_{max} values were enhanced in extracts of the former. These differences between the interactions of araC and dCyd with dCyd kinase may find an explanation in studies which explored the kinetic behaviour (69) and allosteric inhibition (103) of calf thymus dCyd kinase and suggested that araC and dCyd interact with different but interacting sites on this enzyme.

The enhanced K_m value for araC phosphorylating activity by extracts of DU-treated cells relative to that by extracts of untreated cells may be explained in two ways. (1) The relative concentrations of different species of dCyd kinase may change with cell cycle stage, and a type abundant in S phase (stage of cell cycle arrest in the presence of DU) may

possess a lesser affinity for araC relative to other species. However, only one species of dCyd kinase was demonstrable by affinity chromatography of extracts of DU-treated and untreated cells (Fig. 17). Further, Durham and Galanti (46) did not detect differences when dCyd kinase activities of mouse parotid glands in different proliferative states were analyzed by sedimentation techniques. (2) Table 5 illustrates that araCTP and araCDP, as well as araCMP, were reaction products in the assay mixtures assessing the activity of dCyd kinase. Thus, different reactions in the sequence from araC to araCTP may have been rate-limiting in extracts of untreated or DU-treated cells, yielding different K_m parameters for araC phosphorylation.

The HU-induced expansion of araC nucleotide concentrations in RPMI 6410 cells was expressed within 30 min and for at least 12 hr after the provision of HU (Table 3 and Fig. 24). Since others have demonstrated that HU does not reduce cellular concentrations of dCyd phosphates (see Chapter I, Section C), release of dCyd kinase from allosteric inhibition by dCTP does not appear to explain the HU-stimulated araC anabolism. Data presented in this chapter showed that the araC phosphorylating activity of RPMI 6410 cell extracts had the characteristic that both K_m and V_{max} parameters increased during a 4 to 12 hr interval after the addition of HU (Table 6). The HU-dependent enhancement of araC anabolism may thus be the result of RPMI 6410 cell accumulation at a cell cycle locus where dCyd kinase activity is enhanced. Results by

others (120,136) have shown that cells incubated in the presence of HU accumulated at the G_1 -S border.

VII. Breakdown of 1- β -D-Arabinofuranosylcytosine Anabolites

A. Introduction.

Few studies of the cellular catabolism of araC anabolites, the passage of araC into the extracellular medium and drug effects on these processes have been published. The recent demonstration that an increase of the retention time of araC metabolites in murine leukemic tumor cells correlated with greater antitumor activity (140) prompted the evaluation of (1) the catabolism of araC metabolites in RPMI 6410 and HeLa cells and (2) the effect of two inhibitors of nucleoside transport, NBMPR and dipyridamole, on the passage of araC into the extracellular medium. Our desire to define the metabolic fate of the anabolites araCDPcholine and araCDP-ethanolamine, especially with respect to the relevance of these compounds to araC toxicity, further justified this study.

B. Results.

It is apparent in Fig. 26 that the anabolism of araC in RPMI 6410 cells was enhanced during incubation in the presence of DU for 14 hr and that araCDPcholine and araCTP were the principal anabolites of araC, as reported in Appendix B. Fig. 26 also shows that cellular concentrations of araC metabolites including araCTP and araCDPcholine declined with time when the cells were transferred to medium without araC. Cellular concentrations (nmoles/ 10^9 cells) of

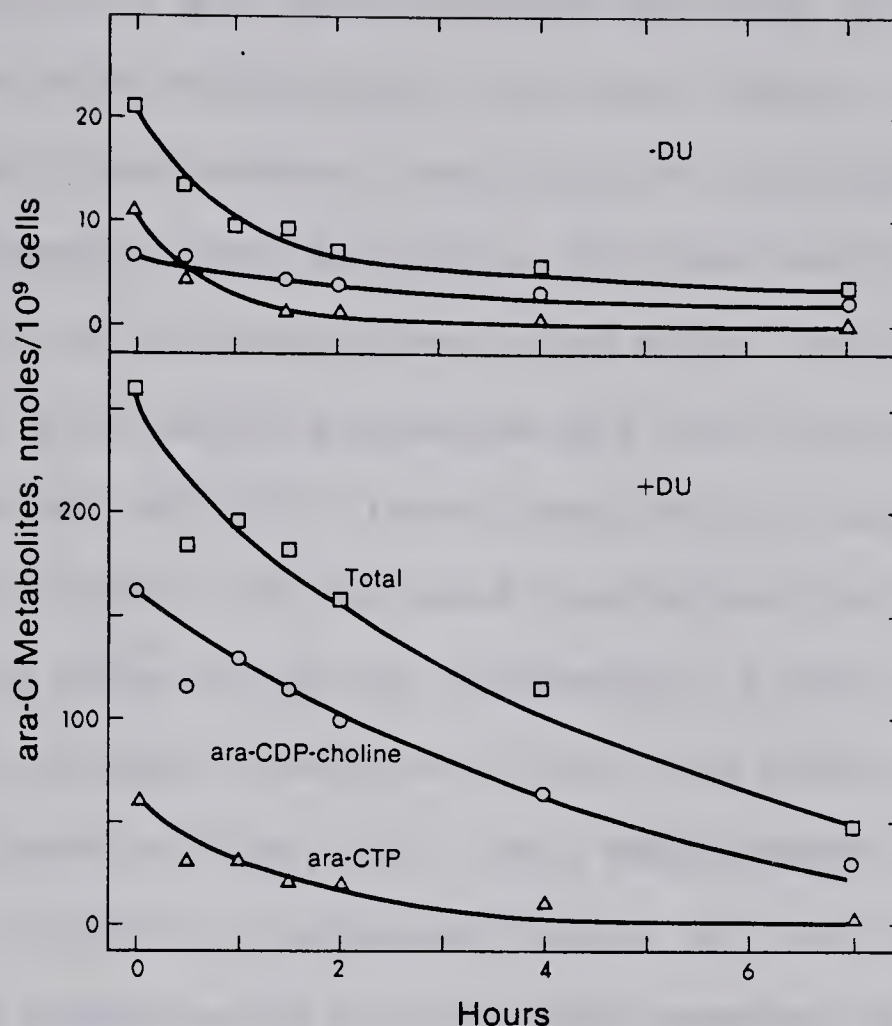


Figure 26. Decay of araC metabolites in RPMI 6410 cells. Cells were incubated for 14 hr in growth medium containing 3 μM [5,6- ^3H]araC (1 $\mu\text{Ci}/\text{ml}$) in the presence or absence of 9 μM DU. Cells were resuspended in drug-free growth medium (zero time) and the cultures sampled at the indicated intervals thereafter. Perchloric acid extracts of the cell samples were analyzed by TLC using solvent 1.

araC metabolites that accumulated during the 14 hr interval of culture in the absence and presence of DU were, respectively, araCTP: 11.1 and 62.0; araCDP choline: 6.7 and 163.4; total acid-soluble metabolites: 21.4 and 262.5.

The decay time courses from Fig. 26 indicated that (1) the decay processes were biphasic, (2) the decline of araCTP and araCDPcholine concentrations were major determinants of the rapid and slow decay processes and (3) the catabolism of araCDPcholine was slightly slower than that of araCTP.

The araC content of the acid-insoluble fraction of the lymphoid cells after 14 hr of incubation in the presence and absence of DU, respectively, was 0.032 and 0.024 nmoles/ 10^9 cells (experiment of Fig. 26). In 2 experiments, it was found that, in spite of enhanced levels of araC anabolites formed in the presence of DU, the araC content of the cellular acid-insoluble fraction did not vary significantly from that of cells incubated in the absence of DU. Thus, the synergism in growth inhibition resulting from the presence together of araC and DU (Chapter III) is apparently not related to araC incorporation into polynucleotides in RPMI 6410 cells.

Labelled compounds which appeared in the extracellular medium during decay of araC metabolites were identified as follows: medium samples were freeze-dried and the residues extracted with cold 0.4 M perchloric acid; the extracts were neutralized and analyzed by TLC using solvents 1 and 3 (Table 1). The disappearance of cellular araC metabolites was accounted for almost completely by the appearance of

araC in the incubation medium. Thirty and 120 min after initiation of the decay process, there was no evidence of araC phosphates in the incubation medium and araU accounted for about 2% of the ^3H content. Rustum (140) reported that the catabolism of araCTP in murine leukemic cells in vitro was accompanied by the appearance of araC in the extracellular medium.

Table 10 lists half-times for the rapid and slow decay of araC metabolites in RPMI 6410 cells and HeLa cells: semilogarithmic plots (81) were drawn of decay time courses performed such that segments representing rapid and slow decay processes were each determined by at least 4 datum points performed in duplicate (RPMI 6410 cells) or triplicate (HeLa cells). Half-times for the rapid and slow processes in RPMI 6410 cells ranged between 17-30 min and 160-276 min, respectively, whether derived from changes in the total acid-soluble radioactivity or from the total cellular araC content. Decay kinetics were similar whether araC anabolites had been accumulated in the presence or absence of DU (Table 10), suggesting that neither DU nor its metabolites influenced the catabolism of araC anabolites significantly.

Fig. 27 illustrates the effects of NBMPR and dipyridamole on the decay of araC metabolites in RPMI 6410 cells. In the presence of 6 μM NBMPR, the decay process was inhibited to a minor extent in several experiments. In contrast, the mediated uptake of araC by RPMI 6410 cells was abolished completely by 5 μM NBMPR in short term assays

TABLE 10

Half-time Periods for the Decay of Cellular Content of AraC Metabolites

Semilogarithmic plots of the exponential time courses of the decay of araC metabolites from RPMI 6410 and HeLa cells were analyzed to obtain half-time periods ($t_{0.5}$) for rapid and slow decay processes under a variety of experimental conditions.

Exp. No.	Cells	Concentration (μ M)				$t_{0.5}$ (min)	
		Accumulation		Decay		Rapid	Slow
		DU	dThd	NBMPR	Dipyridamole		
1.	RPMI 6410 ^a	10	0	0	0	30	276
		0	0	0	0	27	252
2.		10	0	0	0	26	206
		0	0	0	0	23	204
3.		10	0	0	0	17	230
		10	0	6	0	27	289
4.	RPMI 6410 ^b	10	0	0	0	25	186
		10	0	6	0	38	252
5.		10	0	0	0	23	223
		10	0	6	0	26	282
		10	0	0	20	738 ^c	
6.		10	0	0	0	25	207
		10	0	6	0	29	248
		10	0	0	20	546 ^c	
7.		10	0	0	0	22	160
		10	0	6	0	45	303
		10	0	0	20	534 ^c	
8.	HeLa ^b	0	1000	0	0	59	396
9.		0	1000	0	0	52	528
		0	1000	5	0	52	528
10.		0	1000	0	0	45	402
11.		0	1000	0	20	228 ^c	

^aacid-soluble radioactivity was measured

^btotal cellular radioactivity was measured

^csingle component decay curve

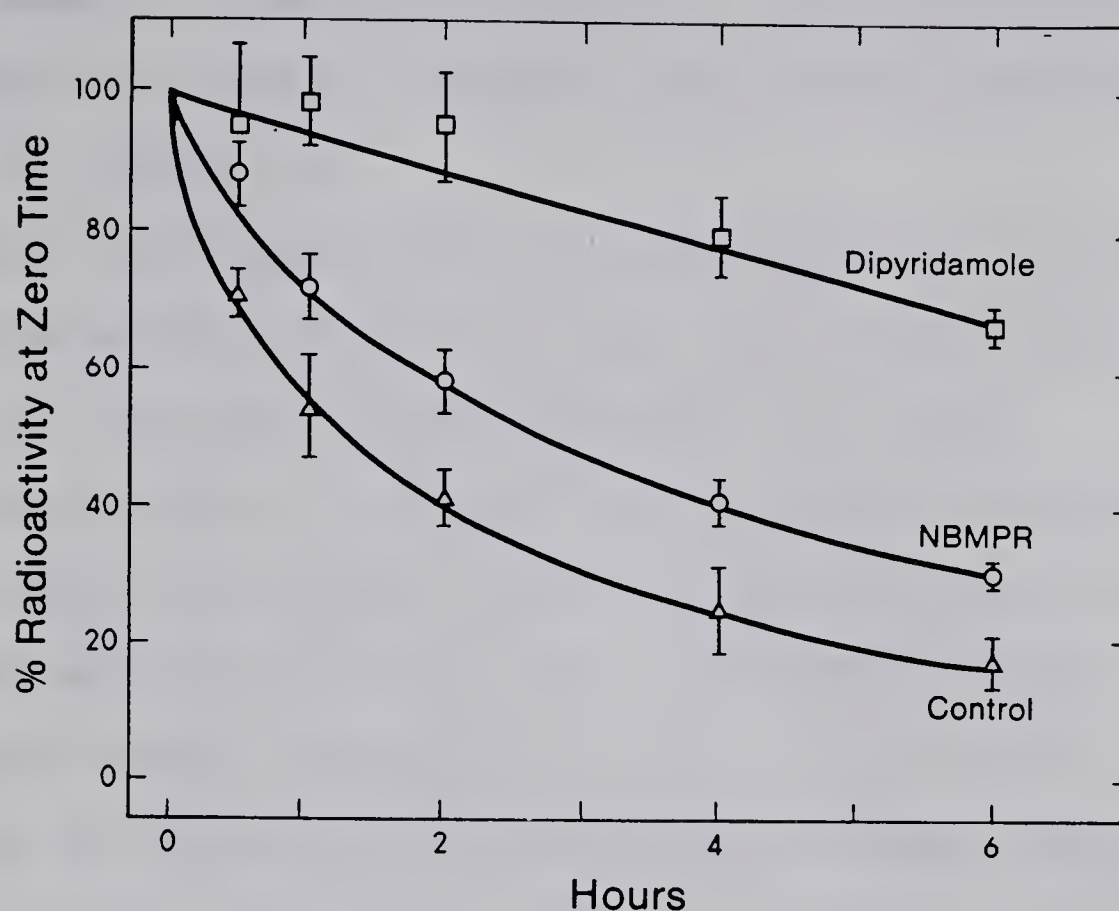


Figure 27. Effects of NBMPR and dipyridamole on the decay of araC metabolites in RPMI 6410 cells. Cells were incubated for 14 hr in growth medium containing 3 μM [5,6- ^3H] araC (1 $\mu\text{Ci/ml}$) and 10 μM DU and then resuspended (zero time) in growth medium without additives (control) or containing 6 μM NBMPR or 20 μM dipyridamole. The cell suspensions were sampled after the intervals shown and total cellular radioactivity was determined. Data points represent averages \pm S.D. from 3 independent experiments in which values for total cellular araC content at zero time were 218, 238 and 195 nmoles/ 10^9 cells.

(Fig. 6). The decay of araC metabolites in the presence of NBMPR was also biphasic (Fig. 27). In the presence of dipyridamole, retention periods for araC metabolites were prolonged and single component decay curves were obtained (Fig. 27, Table 10).

Fig. 28 demonstrates that the presence of 30 μ M araC in the extracellular medium did not significantly alter the decay of araC metabolites from RPMI 6410 cells.

The kinetics of decay of araC metabolites from HeLa cells were also biphasic (Fig. 29) and the final decay product was extracellular araC. The medium content of araC accounted almost completely for the change in the cell content of araC metabolites during the decay interval. The extracellular decay product was identified as araC by the procedure described above for RPMI 6410 cells, after 0.5 and 2 hr of the decay process. There was no evidence of araC phosphates in the extracellular medium and araU accounted for 3% of the recovered activity. As with RPMI 6410 cells, the presence of 30 μ M araC in the extracellular medium did not significantly alter the decay of araC metabolites from HeLa cells (Fig. 30).

The half-times for the rapid and slow decay processes for araC metabolites in HeLa cells, which ranged between 45-59 min and 396-528 min, respectively, were longer than those for RPMI 6410 cells (Table 10). NBMPR did not affect the decay processes in HeLa cells (Fig. 29 and Table 10). In the presence of dipyridamole, retention periods for araC

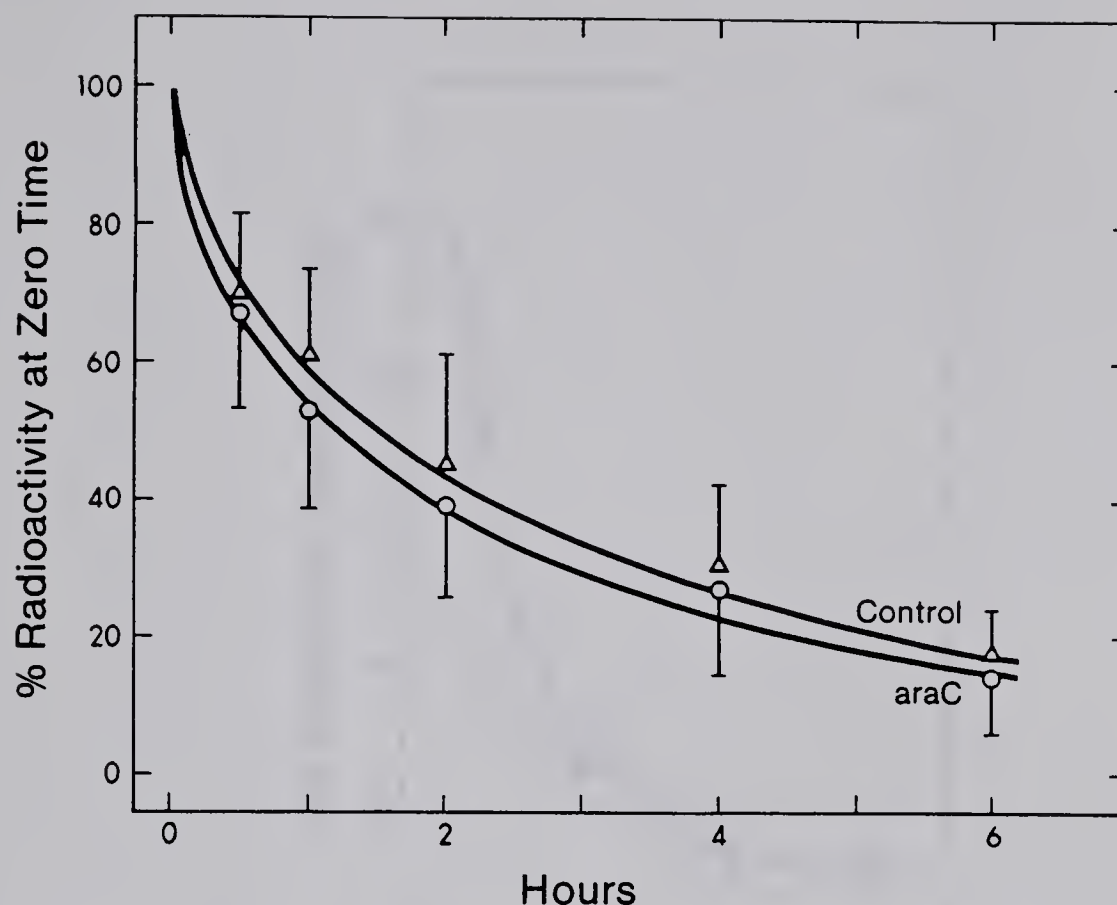


Figure 28. Effect of extracellular araC on the decay of araC metabolites from RPMI 6410 cells. Cells were incubated with araC and DU for 14 hr as described in Fig. 27 and then resuspended (zero time) in growth medium without additives (control) or containing 30 μ M unlabelled araC. The cell suspensions were sampled after the intervals shown and total cellular radioactivity was determined. Data points represent averages \pm S.D. from 3 independent experiments in which values for total cellular content of araC at zero time were 269, 201 and 148 nmoles/ 10^9 cells.

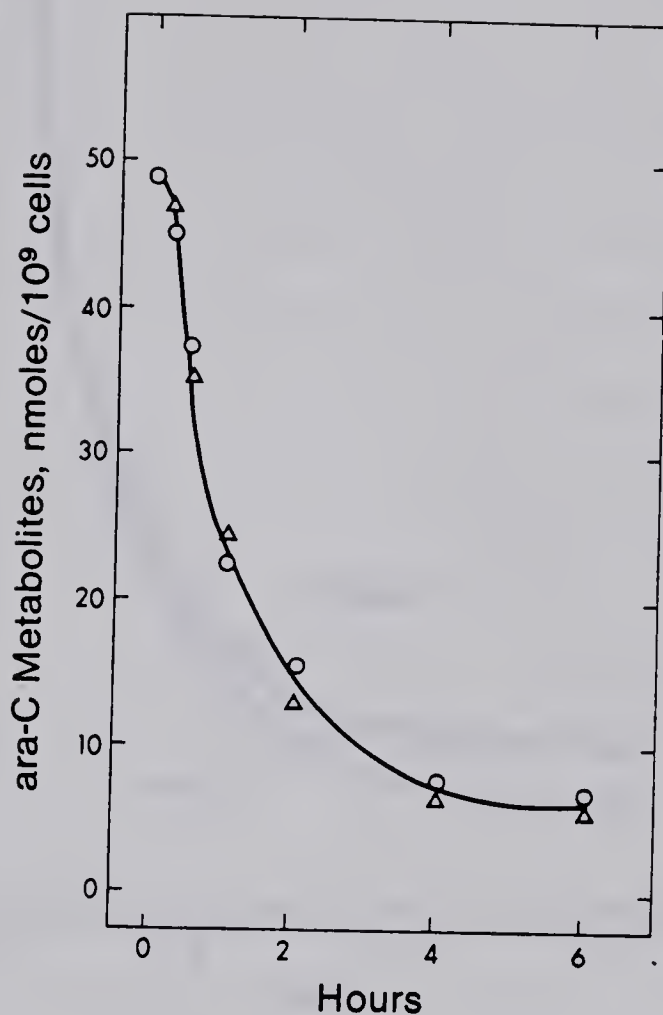


Figure 29. Decay of araC metabolites from HeLa cells. Replicate monolayer cultures were incubated for 6 hr in MEM containing 1 mM dThd and 0.6 μM [5,6- ^3H]araC (1 $\mu\text{Ci/ml}$). The monolayer cultures were then rinsed and at zero time, 2 ml of medium with (O) or without (Δ) 5 μM NBMPR was added. At intervals, total cellular radioactivity was determined. Data points represent averages of triplicate determinations.

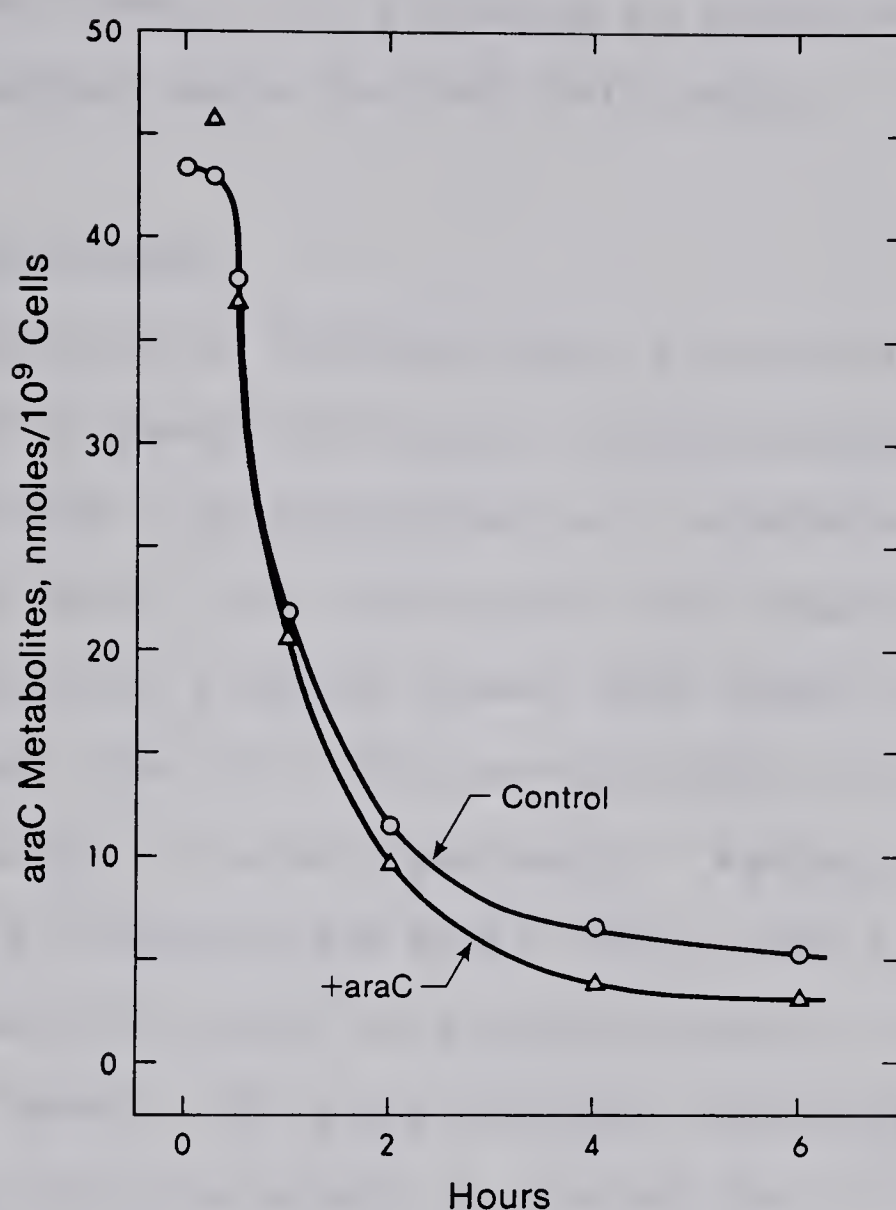
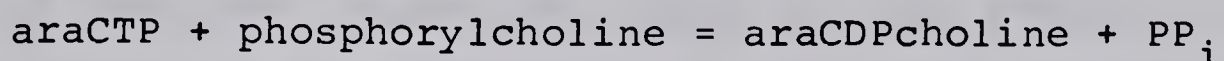


Figure 30. Effect of extracellular araC on the decay of araC metabolites from HeLa cells. Replicate monolayer cultures were incubated in the presence of dThd and araC for 6 hr as described in Fig. 29. The monolayer cultures were then rinsed and at zero time, 2 ml of medium without additives (control) or containing 30 μ M unlabelled araC was added. Data points represent averages of triplicate determinations. Decay in the presence of 100 μ M unlabelled araC yielded similar results.

metabolites were increased and single component decay curves were obtained (Table 10), a finding in accord with the results presented above for RPMI 6410 cells.

C. Discussion

The formation of araCDPcholine, a reversible reaction, is catalyzed by phosphorylcholine cytidylyltransferase (76):



In RPMI 6410 cells, time courses for the catabolism of araCDPcholine were slightly slower than those for araCTP (Fig. 26) and, therefore, the reversibility of this reaction was not apparent in these experiments. Pathways for the catabolism of araCDPcholine might include the following:

(1) conversion to araCTP by phosphorylcholine cytidyltransferase (see above), (2) group transfer reactions in which the phosphorylcholine moiety is donated [as by the action of CDP:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2)] or (3) enzymatic hydrolysis of the pyrophosphate linkage, as by apyrase (see Appendix B), to yield araCMP and phosphorylcholine. The breakdown possibilities for araCTP include (i) hydrolysis by pyrophosphatases or (2) phosphate transfer reactions as by nucleotide monophosphate or diphosphate phosphotransferases (see Fig. 2). AraCMP yielded by the various pathways would be subject to hydrolysis by 5'-nucleotidase (Fig. 2) to produce araC, the major catabolic product in the extracellular medium. The remainder of this section discusses the mechanism by which intracellular araC

crosses the plasma membrane to reach the extracellular fluid.

The minor inhibition by NBMPR of araC anabolite decay processes in both HeLa cells (Fig 29) and RPMI 6410 cells (Fig. 27) at concentrations that eliminate inward transport of araC suggests the possibilities that (1) the nucleoside transport mechanism may not participate in the outward movement of the final catabolite, araC (2) that NBMPR did not enter cells and (3) NBMPR bound to the outside aspect of the transporter does not block flux from inside to outside. In contrast, Cass and Paterson (26) have shown that NBMPR bound to the outside aspect of the nucleoside transporter blocks the efflux of Urd and dThd from "loaded" red blood cells. From present results, it would appear that, to block nucleoside efflux (inside to outside) from RPMI 6410 and HeLa cells, NBMPR must bind to inhibitor sites on the inner aspect of the plasma membrane and that such sites are not readily accessible to extracellular NBMPR.

Dipyridamole, a potent inhibitor of nucleoside transport, clearly prolonged the retention of araC metabolites by both RPMI 6410 and HeLa cells; dipyridamole competes with NBMPR (K_i about 30 nM) for binding to the high affinity inhibitor sites on the nucleoside transport mechanism of HeLa cells (124). The dipyridamole results suggest that (1) araC outflow is mediated, (2) inhibitory sites are present on the inner aspect of the nucleoside transporter and (3) external NBMPR may not have access to the latter. However, dipyrid-

amole effects on permeation processes are not specific for nucleosides (6) and inhibition by dipyridamole of the diffusional uptake (i.e., non-mediated) by cells of L-glucose and cytosine has been demonstrated (56). Thus, the argument for a transporter mediated outflow of araC is tentative.

The observation that time courses of the catabolism of cellular araC anabolites were not influenced by extracellular araC concentrations of 30-100 μM indicates a clear separation of the anabolic (influx) and catabolic (efflux) processes.

The present results suggested that dipyridamole-induced inhibition of anabolite degradation might enhance the cytotoxicity of araC. This idea was tested by attempting to enhance the toxicity of araC toward RPMI 6410 cells as follows: cells were cultured with 3 μM , 10 μM or 100 μM araC for 12, 4 or 1 hr, respectively, and then transferred to medium with or without 20 μM dipyridamole for continued culture. Cell proliferation rates in dipyridamole-containing medium were not different from those in the absence of dipyridamole, indicating that the latter did not influence araC cytotoxicity.

VIII. Chemotherapy of Murine Neoplasms with AraC in Combination with other Drugs

A. Introduction.

The enhancement of araC uptake by DU and HU suggested that combinations of these drugs with araC might enhance the latter's therapeutic effects. Rustum (140) showed that the therapeutic effectiveness of araC against murine neoplasms was related to the concentrations of araCTP achieved in the neoplastic cells. To test these ideas, toxicity to mice of araC-DU and ara-HU combinations was measured and such combinations were employed in the treatment of several transplanted mouse neoplasms.

B. Results.

1. Drug Sequence-Dependent Toxicity in Mice Treated with Low Doses of AraC and DU.

The dosage causing 10% lethality when araC was administered i.p. once daily for 5 days was 310 (258-372)* mg/kg, as determined on groups of 10 female BDF₁ mice**. The corresponding LD₁₀ for DU was 190 mg/kg***.

*95% confidence limits calculated by the method of Litchfield and Wilcoxon (89).

**Data from Dr. E.S. Jakobs of this Laboratory, mice were treated with araC at dosages of 200, 400, 500 and 800 mg/kg; the LD₁₀ value was derived from a plot of log dosage against probit mortality during days 1 to 14.

***Report MRIKC-PT-3899-74-02, Laboratory of Toxicology, National Cancer Institute, Bethesda, Md.

During attempts to apply the biochemical findings described herein to the treatment of leukemic mice, a remarkable toxicity was evident when injections with low dosages of DU (10 mg/kg) were followed by araC (10 mg/kg). Table 11 summarizes a sequence-interval study which shows that when the administration of DU (10 mg/kg) preceded that of araC (10 mg/kg), deaths occurred within 4 to 8 days and the toxicity of the treatment was markedly influenced by the interval between the drug injections. When the drugs were administered together, the treatment was tolerated with no significant weight loss; however, when DU preceded araC by as little as 2 hr, the treatment became highly toxic. The same dosages and intervals were tolerated with minor weight loss when the drug sequence was reversed. In the first and last treatment regimens listed in Table 11, DU and araC treatments were alternated at 12 hr intervals, but the first regimen began with DU and the last with araC. The toxicity of the first regimen was greater (30% mortality) than the latter (no mortality). Damage to the intestinal epithelium has now been shown to be the cause of death in mice treated with lethal DU-araC combinations (122). The experiment of Table 11 was performed by Dr. E.S. Jakobs of this Laboratory.

2. Activity of Low Doses of AraC and DU Against the Murine Leukemia L1210.

DU-araC combinations were employed in the treatment of BDF₁ mice bearing i.p. implants of leukemia L1210 cells; low

TABLE 11

Sequence-dependent lethality of DU-araC combinations

Groups of 8 or 9 BDF₁ female mice were treated with DU (10 mg/kg) and araC (10 mg/kg) in the sequence-interval pattern specified below; drug injections were based upon individual animal weights at the time of treatment. The combined treatments were repeated at 24 hr intervals to a total of 5 treatments; treatments were begun between 8:30 and 9:00 a.m. and survivors were recorded daily.

Treatment			Number alive on day ^a						Mean weight loss by day 5 (%)
1st	Interval (hr)	2nd	4	5	6	7	8	30	
DU	12	araC	9	9	9	6	6	6	15
DU	8	araC	8	8	7	4	4	4	21
DU	6	araC	7 ^b	6	4	0			20
DU	4	araC	8	4	2	0			24
DU	2	araC	8	7	4	2	1	1	18
DU	0	araC	8	8	8	8	8	8	1
araC	2	DU	8	8	8	8	8	8	3
araC	4	DU	8	8	8	8	8	8	6
araC	6	DU	8	8	8	8	8	8	3
araC	8	DU	8	8	8	8	8	8	9
araC	12	DU	8	8	8	8	8	8	12

^a Days were defined as successive 24 hr intervals after the 1st drug injection; survivors were recorded during the 6th hr of each such day.

^b Initial number was 8; 1 mouse died on day 4.

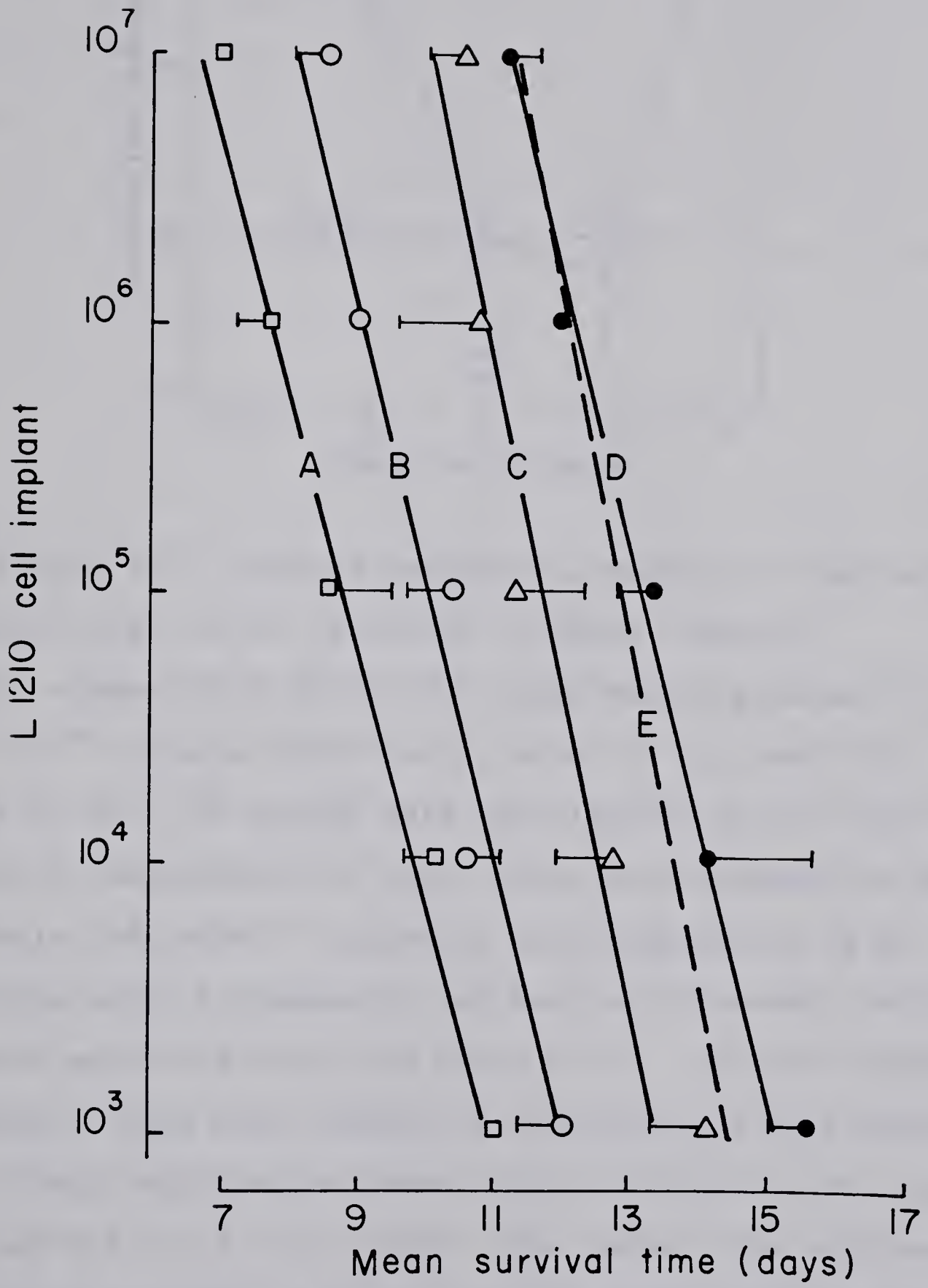
dosages were employed because of the host toxicity encountered when DU was administered prior to araC (Table 11). Fig. 31 illustrates (1) the relationship between host survival time and the number of leukemia L1210 cells implanted in female BDF₁ mice, and (2) the effect on survival time of DU (3 mg/kg), araC (10 mg/kg) and DU-araC combination (DU followed 4.5 hr later by araC) at these dosages. It is evident that the effect of the drug combination was not significantly different from the summed effect of the individual drugs.

3. Activity of AraC-HU Combinations Against the Murine Leukemia L1210.

The toxicity of araC-HU combinations toward BDF₁ female mice was sequence dependent: when administration of HU (500 mg/kg) preceded that of araC (20 mg/kg) by 6 hr in 5 consecutive daily treatments, 2 of 6 mice died from drug toxicity by day 8. When araC administration preceded that of HU by 6 hr at these dosages, 5 of 6 mice died from drug toxicity. No deaths were observed when both agents were administered simultaneously. When combinations of HU (100 mg/kg) and araC (20 mg/kg) were administered daily for 5 treatments, no deaths or significant weight changes occurred with intervals between injections of 0, 2, 4, or 6 hr, whether HU or araC was administered first.

The experiment of Fig. 32 examined the sequence dependence of therapeutic effects with araC-HU combinations employed in the treatment of mice bearing leukemia L1210. Simultaneous administration of the two agents daily

Figure 31. Treatment of leukemic mice with low doses of DU and araC. Groups of 5 female BDF₁ mice were implanted i.p. with graded numbers of leukemia L1210 cells; after 24 hr, DU (3 mg/kg) or araC (10 mg/kg) were administered i.p. as single agents (B and C, respectively), or in a combination treatment in which DU was administered 4.5 hr prior to araC (D). Five such treatments were administered at 24 hr intervals. Control mice (A) were treated with 0.15 M NaCl. Plot E represents results expected if the effects of the agents in combination were exerted independently.



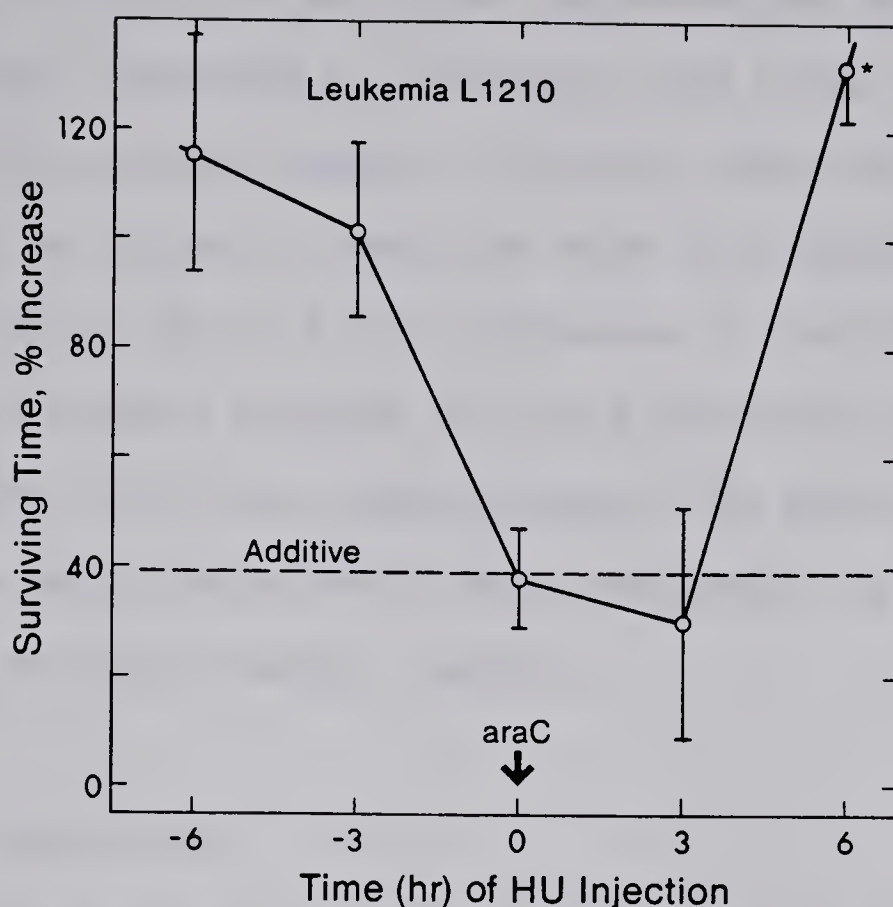


Figure 32. Sequence dependent activity of combinations of HU and araC in the treatment of mouse leukemia L1210. Groups of 10 female BDF₁ mice were implanted ip with 0.95×10^6 leukemia L1210 cells; after 24 hr, araC (20 mg/kg) or HU (200 mg/kg) were administered ip as single agents or combinations in which drugs were injected at the intervals indicated. Treatments were repeated at 24 hr intervals until 4 treatments had been administered; survival was then monitored daily and means \pm S.D. (vertical bars) are shown. Days were defined as successive 24 hr intervals after tumor implantation (time zero); saline-treated controls survived 6.6 ± 1.4 (S.D.) days. The dotted line represents the additive effect of HU and araC as single agents; the latter agents yielded 16.7 and 22.7% increases of survival time, respectively.

*1 mouse died of drug toxicity

for 4 days starting 24 hr after implantation of leukemic cells yielded increases of survival time equal to the summed effects of the single agents. However, when HU was administered 3 to 6 hr prior to araC, or when araC administration preceded that of HU by 6 hr, increases in survival time exceeded the summed effects of the individual agents; in contrast, when araC was administered 3 hr prior to HU, approximate additive effects were observed. A confirmatory experiment yielded similar results.

C. Discussion

The stimulation of araC anabolism by DU does not appear to have application to the therapy of leukemia L1210 because of the profound and limiting host toxicity of regimens in which DU was administered prior to araC (Table 11). When doses of DU and araC acceptable with respect to host toxicity were administered to leukemic mice, no therapeutic interaction of DU and araC was discernable (Fig. 31). Hence, the sensitization to araC by DU of a vital tissue in the mouse (evidently the small intestine) was achieved at lower drug dosages than that which might have been achieved in the tumor cells. In view of our results, the clinical use of DU-araC combinations should be approached with great caution.

Schedule-dependence was demonstrable for the therapeutic effectiveness of HU-araC combinations toward leukemia L1210 cells (Fig. 32). We suggest that the basis of the HU influence on the toxicity of araC toward tumor cells

may involve, at least in part, the HU-dependent enhancement of araC uptake (Chapter V). The partial degree of cell cycle synchrony induced by HU in leukemia L1210 cells in vivo (113) may likewise contribute to the enhanced araC toxicity following HU pretreatment.

The mechanism by which administration of araC influences the therapeutic effect of subsequently administered HU (Fig. 32) is less clear, but the following observations are provided: (a) the cytotoxicity toward leukemia L1210 cells of HU administered after araC was interval-dependent (Fig. 32), intervals greater than 3 hr being necessary for synergistic interaction between araC and HU, and (b) host toxicity of HU administered 6 hr after araC (20 mg/kg) was dependent on the dosage of HU, 100 and 500 mg/kg being lethal in 0 and 83% of animals, respectively. Neil and Homan (113) have showed that maximum in vivo synchrony of leukemia L1210 cells in S phase occurred 8 to 10 hr after i.p. administration of araC (10-100 mg/kg), with a small and larger proportion of cells being in S phase 3 and 6 hr after araC injection, respectively. Cytotoxicity by HU has been shown to be S phase dependent (148).

The schedule dependence of combination chemotherapy of the murine leukemia L1210 with araC and 5-azacytidine was investigated by Neil and coworkers (112): antagonism was observed when both drugs were administered simultaneously. The most effective mode, with respect to enhancing the survival time of the leukemic mice, involved the administration of 5-azacytidine 8 to 10 hr after that of araC. LePage and

White (88) likewise showed that mice treated simultaneously with araC and 6-thioguanine were protected from the lethal effects of the latter.

IX. GENERAL DISCUSSION

The present work investigated the metabolism of araC in a variety of cell types in the absence of other additives and in the presence of DU, HU or high concentrations of dThd. The findings are discussed briefly below under 5 headings.

A. AraCDPcholine and AraCDPethanolamine

When cultures of RPMI 6410, HeLa, LS and L5178Y cells and of circulating leukemic leukocytes from AML patients were incubated with araC for 1 hr or longer, the formation of araCDPcholine and araCDPethanolamine was evident. The formation of araCTP preceeded that of araCDPcholine in accordance with the synthesis of endogenous CDPcholine by phosphorylcholine cytidyltransferase. When DU or PF were present, the proportion of the araC-associated cellular radioactivity present as araCDPcholine and araCDPethanolamine was increased, possibly because depletion of CTP concentrations by DU and PF (see Chapter I) precluded the competition of CTP with araCTP for phosphorylcholine cytidyltransferase and phosphorylethanolamine cytidyltransferase.

Whether araCDPcholine and araCDPethanolamine will serve as substrates in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine, respectively, remains unknown, as does the significance of these araC metabolites to araC lethality. In this respect, investigations of the metabolism of araCDPcholine (labelled with different isotopes in

the base and choline moieties) by tissue extracts (75) and whole cells (see below) would be a most logical continuation of this project. Phospholipid vesicles (119,134) might be used to introduce araCDPcholine* into intact cells. Alternatively, cells rendered permeable to nucleotides, as by treatment with lysolecithin (96) might be used to study the cellular metabolism of araCDPcholine or araCDPethanolamine.

B. Inhibition of RPMI 6410 Cell Proliferation by
DU and AraC.

The lethal effects toward RPMI 6410 cells cultured in the presence of araC and DU together for 24 hr was 10-fold greater than the sum of the independent effects of each agent alone. Also, the anabolism of araC in these cells was enhanced several-fold when DU was present. RPMI 6410 cells pretreated with DU (1) were "sensitized" to the lethal action of araC and (2) took up more araC in short term assays than did untreated cells. It was also shown that DU blocked the cell cycle progression of RPMI 6410 cells at a locus in S phase. Collectively, these results imply that DU "sensitized" RPMI 6410 cells to araC toxicity by (1) increasing cellular concentrations of araC phosphates and (2)

*We have observed that araCDPcholine does not enter cells without prior hydrolysis to araC.

stopping cells in S phase of the cell cycle. Cells are more susceptible to araC lethality in S phase than in other phases of the replication cycle.

C. Drug-Enhanced Anabolism of AraC.

Treatment with DU, HU and high concentrations of dThd enhanced the formation of araC phosphates in RPMI 6410, HeLa and L5178Y cells. Plagemann and coworkers (133) have recently reported similar findings with rat hepatoma cells. In the present study none of these compounds enhanced araC uptake by peripheral leukocytes from leukemia patients during incubation at concentrations which consistently enhanced araC metabolism in cultured cells.

When RPMI 6410 cells were cultured in the presence of DU, cell cycle progression was blocked in S phase and extracts of cells from such cultures (rich in S phase cells) contained more araC phosphorylating activity than did extracts from untreated cells. Others have shown that the activity of dCyd kinase is greatest during S phase of the cell cycle (Chapter I, Section G). Extracts of HU-treated RPMI 6410 cells also contained more araC phosphorylating activity than did extracts of untreated cells. The presence of HU (2,120,136,148) or dThd (153) blocked the progression of cultured cells through the cell cycle at the G_1 -S border, while S period cells remained trapped in S.

It is proposed that the mechanism responsible for the enhancement of araC anabolism by DU, HU and dThd involves

the trapping and accumulation of S phase cells with increased araC phosphorylating activity. In our studies, the proportion of human peripheral leukocytes (from leukemic patients) which synthesized DNA, and thus were "in cycle", was small (0.5 to 8.5%); the mechanism proposed above provides a basis for the failure of DU, HU and dThd to enhance araC uptake in these cells.

Feedback inhibition of dCyd kinase by dCTP has been demonstrated repeatedly in broken cell preparations (28, 69, 77, 103) but to the author's knowledge, no such demonstration has been shown in intact cells. Plagemann and coworkers (133) have proposed that the stimulatory influence of DU and dThd on araC uptake results from the depletion of cellular dCTP concentrations and consequent stimulation of dCyd kinase activity in the presence of these drugs. The stimulation of araC anabolism by HU, which does not appear to reduce cellular dCTP concentrations (see Chapter I, Section C), cannot be explained by such a feedback inhibition mechanism. Present results do not permit us to estimate the contribution of dCyd kinase release from feedback inhibition by dCTP to the enhancement of araC phosphate formation in cells incubated with DU or dThd. With respect to the latter comment, reliable determination of deoxyribonucleotide concentrations in cells incubated with DU, HU or dThd would aid interpretation of the present results.

D. Breakdown of araC Anabolites.

When RPMI 6410 cells or HeLa cells were incubated with araC and then transferred into growth medium without araC, the cellular concentration of total araC metabolites declined according to a biphasic process. In RPMI 6410 cells, half-times for the rapid and slow decay processes ranged between 17-30 min and 160-276 min, respectively. The appearance of araC in the extracellular fluid accompanied the decline of cellular araC metabolites.

NBMPR inhibited only slightly the catabolism of araC metabolites in RPMI 6410 cells, while that in HeLa cells was unchanged in the presence of NBMPR. It was proposed (Chapter VII) that (1) binding of NBMPR to inhibitory sites on the outer aspect of the nucleoside transporter did not influence the efflux of araC and (2) intracellular inhibitory sites were not readily accessible to extracellularly provided NBMPR. The existence of inhibitory sites for NBMPR on the inner aspect of the plasma membrane was implied by the following observations: (1) dipyridamole inhibited the breakdown of cellular araC anabolites and (2) dipyridamole competed with NBMPR for binding to high affinity inhibitory sites on HeLa cells (124). For reasons outlined in Chapter VII, the suggestion that the outward passage of araC is mediated remains tentative.

Futher experiments should attempt to take advantage of the inhibitory influence of dipyridamole on the breakdown of araC metabolites and passage of araC into extracellular fluids to enhance (1) the lethal action of araC against cultured cells and (2) the effects of therapy of murine neoplasms with araC.

E. Combinations of AraC with DU or HU Against a Murine Neoplasm.

When attempts were made to apply the biochemical findings described herein to the treatment of leukemic mice with DU-araC combinations, a profound host toxicity was observed when DU was administered prior to araC. Concentrations of drugs (DU administered before araC) which circumvented this toxicity produced no interaction between DU and araC with respect to the lethality of leukemic cells.

The host toxicity of HU-araC combinations was less profound and an interesting sequence dependence was observed regarding the increase in life span of leukemic mice treated with HU-araC combinations. When HU was administered 3 or 6 hr prior to araC or 6 hr after araC, the therapeutic effects (increases of life span) were several-fold greater than the sum of the effects of HU or araC given alone.

These observations were explained on the basis of (1) the HU-dependent enhancement of araC anabolism and (2) partial cell cycle synchrony achieved in the presence of araC or HU and the susceptibility of S phase cells to the lethal

effects of both these drugs.

F. General Comments.

In the opening chapter (Section H), the aim of the present work was defined as follows:

"...to enhance the cytotoxicity of araC by combining its administration with that of other drugs. To achieve that end, we investigated the anabolism and catabolism of araC in sensitive cells in tissue culture and in mice and assessed the influence of other drugs upon these processes."

These goals have been realized in part: agents were found which stimulated araC anabolism (e.g. DU, HU, dThd) and another which inhibited araC catabolism (i.e. dipyridamole). The cytotoxicity of araC toward RPMI 6410 cells was enhanced by DU-pretreatment while the treatment of leukemic mice with araC was several-fold more effective following pretreatment of the animals with HU. The application of the inhibitory influence of dipyridamole on araC catabolism to the treatment of neoplasms remain to be explored.

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APPENDIX A

Binding of the Nucleoside Transport Inhibitor Nitrobenzylthioinosine to HeLa Cells

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SUMMARY

LAUZON, GILLES J. & PATERSON, ALAN R. P. (1977) Binding of the nucleoside transport inhibitor nitrobenzylthioinosine to HeLa cells. *Mol. Pharmacol.*, 13, 883-891.

Nitrobenzylthioinosine (NBMPR), a potent inhibitor of nucleoside transport, was bound tightly but reversibly to HeLa cell membrane sites associated with the nucleoside transport mechanism. Site-specific binding was assayed with [³⁵S]NBMPR and a competing, nonisotopic congener. Mass law analysis of the binding data indicated that each HeLa cell possessed about 10⁵ binding sites of a single class which bound NBMPR tightly; the bound inhibitor had a dissociation constant of about 0.1 nM. Occupancy of these binding sites by NBMPR correlated with inhibition of uridine and thymidine uptake; however, the relationship between these parameters was not simple because, as binding saturation was approached (at about 5 nM NBMPR), a substantial fraction (25-30%) of the transport capability remained active but inhibitable by 5 μM NBMPR.

INTRODUCTION

The passage of nucleosides across the plasma membrane of animal cells is mediated by specific elements of the membrane (1-6). The transport¹ of uridine and thymidine by human erythrocytes has been identified as a classical "facilitated diffusion" process; because of the inability of these cells to metabolize uridine or thymidine, it has been possible to demonstrate transport phenomena that involve internal nucleoside pools [such as equilibrium exchange diffusion and accelerative exchange diffusion (2, 3)] and are characteristic of facilitated diffusion. The naturally occurring nucleosides are metabo-

lized upon entering most cells, and it is not known whether transport mechanisms, such as facilitated diffusion, ordinarily function independently of enzymes involved in nucleoside metabolism. The principal approach to the study of nucleoside permeation has been through measurement of initial rates of uptake; kinetic studies have shown that a rate-limiting step in the uptake process is mediated, and it has been assumed frequently that transport is that step.

Accelerative exchange diffusion data have indicated that human erythrocytes have a single type of nucleoside transport mechanism that accepts ribosides and deoxyribosides of both purines and pyrimidines (1-3). In contrast, HeLa cells appear to have several distinct nucleoside uptake mechanisms; the uptake processes for uridine, thymidine, adenosine, and guanosine are distinguishable by kinetic and

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¹ "Transport" refers to the mediated passage of permeant across the plasma membrane of cells; the "uptake" process includes both transport and intracellular metabolism of permeant.

other criteria² (4), and therefore each is separate or, at least, has a step that is distinct from the others.

NBMPR,³ NBTGR, and related compounds are potent inhibitors of nucleoside transport in erythrocytes (2, 3, 7). Erythrocytes were found to have high-affinity binding sites of a single type on the plasma membrane (8, 9); these sites, which were present to the extent of $1.0\text{--}1.5 \times 10^4/\text{cell}$, bound NBMPR with an apparent dissociation constant of 1 nM (9). The sites were presumed to be part of the nucleoside transport mechanism, since inhibition of uridine transport was proportional to the number occupied by NBMPR (9). While transport permeants compete with NBMPR for occupancy of the erythrocyte binding sites (10), there are cogent reasons for believing that the NBMPR binding sites are distinct from the nucleoside permeation sites (10).

NBMPR is also a potent inhibitor of nucleoside uptake in various types of cells which metabolize nucleosides, including HeLa cells (4, 11). Since the thymidine and uridine kinase activities of HeLa cell extracts are unaffected by NBMPR at concentrations well in excess of those effective in blocking nucleoside transport (4), the latter effect is evidently due to impairment of transport. The present report describes the binding of [³⁵S]NBMPR to HeLa cells and explores relationships between binding and inhibition of uridine and thymidine uptake.

METHODS

Chemicals. NBMPR and NBTGR were prepared by established methods (12), using thioinosine and thioguanosine generously provided by Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md.

² A. R. P. Paterson and C. E. Cass, unpublished observations.

³ The abbreviations used are: NBMPR (or nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; thioinosine, 6-thio-9-β-D-ribofuranosylpurine; thioguanosine, 2-amino-6-thio-9-β-D-ribofuranosylpurine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

In the preparation of [³⁵S]NBMPR, the sulfur atom of thioinosine was first labeled by exchange (13) and then alkylated with 4-nitrobenzyl bromide (12). In a typical preparation, 10 μmoles of thioinosine, 0.36 mg of elemental ³⁵S (600 mCi/milliatom, Amersham/Searle, Oakville, Ont.), and 7 ml of freshly distilled, dry pyridine were refluxed with stirring under nitrogen for 1 hr. The ³⁵S exchange was complete by 30 min. The pyridine was evaporated under a stream of nitrogen, and the product was dissolved in 0.5 ml of freshly distilled, dry dimethylformamide containing 20 μmoles of 4-nitrobenzyl bromide; the alkylation reaction (10 min, 20°) was essentially quantitative. NBMPR was isolated by chromatography on 250-μm layers of silica gel (G-HR, Macherey, Nagel and Company). Prior to use, thin-layer plates were washed once with 15% (v/v) methanol in chloroform and heated at 120° for 12 hr. Chromatograms were developed in 15% methanol-chloroform, and the NBMPR band was eluted from the silica gel with methanol. In a typical preparation, [³⁵S]NBMPR (specific activity, 6.6×10^8 cpm/μmole) was obtained in 69% yield (in terms of thioinosine). The labeled product co-chromatographed with NBMPR on paper in these solvent systems: isopropyl alcohol-ammonia-water (70:5:25, v/v), *R_f* 0.79; 5% (w/v) disodium phosphate in water, *R_f* 0.05; and isobutyl alcohol-acetic acid-water (120:30:50, v/v), *R_f* 0.82. When stored in absolute methanol at -20°, [³⁵S]NBMPR preparations were stable for 5 months.

Cell culture. HeLa S3 cells were maintained by weekly passage of monolayer cultures in Eagle's minimal essential medium supplemented with 10% calf serum and 2 mM HEPES buffer (pH 7.4) at 37° in 5% CO₂-air. After six to eight serial passages, cultures were restarted from stocks kept in liquid nitrogen. Spinner cultures, started weekly from trypsinized monolayers, employed calcium-free minimal essential medium supplemented with 5% calf serum and 2 mM HEPES buffer (spinner medium); cell concentrations were kept below 6×10^5 cells/ml and, under these conditions, cell proliferation was exponential

with doubling times of 22-24 hr. Cell culture materials were purchased from Grand Island Biological Company, Calgary.

Binding studies. Binding studies employed cells from spinner cultures collected by centrifugation ($150 \times g$, 5 min) and resuspended in fresh spinner medium at 4×10^6 cells/ml. Binding assays were initiated by mixing equal volumes of cell suspension and calcium-free minimal essential medium containing [^{35}S]NBMPR; assay intervals were terminated by centrifuging ($500 \times g$, 3 min) samples containing at least 10^7 cells. Supernatants were reserved for determination of ^{35}S content using Bray's counting solution (14) and liquid scintillation counting. Cell pellets were thoroughly drained, dissolved in 1.5 ml of NCS tissue solubilizer (Amersham/Searle), and transferred with rinsing into 18 ml of Bray's counting solution. Specific binding was defined as the difference between [^{35}S]NBMPR bound to cells in the absence and presence of NBTGR, the latter at a concentration which displaced bound NBMPR from the cellular binding sites. All assays of ^{35}S activity were performed under the same conditions as those for determination of cell-bound ^{35}S .

Uptake studies. In these experiments, the effect of prior incubation with [^{35}S]NBMPR on the cellular uptake of uridine and thymidine was studied. Cell suspensions, $1-2 \times 10^6$ cells/ml in "uptake medium" (calcium-free minimal essential medium containing 2.5% calf serum and 1 mM HEPES, pH 7.4), were incubated at 20° for 5 min with and without [^{35}S]NBMPR. Uptake assays were then initiated by the addition of [^3H]nucleoside (Amersham/Searle) and terminated by transferring 1.0-ml samples of the incubation mixture into 40 ml of cold buffered NaCl (15) containing $5 \mu\text{M}$ NBTGR. When uptake of [*methyl*- ^3H]thymidine was assayed, the buffered NaCl contained $100 \mu\text{M}$ thymidine. After centrifugation ($500 \times g$, 3 min), cell pellets from assay samples were dissolved in 0.3 ml of NCS tissue solubilizer for assay of ^3H , using Bray's solution and liquid scintillation counting. Permeant uptake by cell samples added directly to 40 ml of cold stopping solution containing

permeant was 30-80 cpm. The [^{35}S]NBMPR content of samples assayed for uptake of ^3H -labeled permeant was small (less than 80 cpm) under the conditions of the liquid scintillation assay for ^3H activity and was corrected for by subtracting appropriate blanks. In parallel samples, the amount of [^{35}S]NBMPR specifically bound to $1-2 \times 10^7$ cells was determined as described above.

RESULTS

Binding of NBMPR to HeLa cells. The present study showed that HeLa cells possess binding sites with high affinity for NBMPR, resembling those on erythrocytes (8, 9). When these binding sites are occupied by NBMPR, nucleoside transport is impaired in either cell type. The distribution of [^{35}S]NBMPR between HeLa cells and the suspending medium was studied as follows. Cells were assayed for ^{35}S content without washing; the difference between the ^{35}S content of cell pellets from incubation mixtures with and without NBTGR (a tightly bound homologue of NBMPR) measured site-specific binding of NBMPR and, at the same time, corrected for ^{35}S attributable to the medium content of the pellets. The specific activity of our [^{35}S]NBMPR preparations, together with the low number of specific binding sites present on HeLa cells, required that at least 10^7 cells be present in each binding assay.

Binding of NBMPR at 37° was almost complete by 1 min (Fig. 1). These data also illustrate the large reduction in NBMPR binding which resulted from prior treatment of cells with NBTGR; this difference was the basis of the assay for site-specific NBMPR binding. NBTGR and NBMPR are both potent, firmly bound inhibitors of nucleoside transport with similar concentration-effect relationships for inhibition of uridine and thymidine uptake² (16).

Dissociation of NBMPR from cellular binding sites was examined in the experiment of Fig. 2. Cells were first labeled by incubation with [^{35}S]NBMPR; NBTGR was then added to the incubation mixture, and cells were assayed at intervals for ^{35}S . At 37° , displacement of [^{35}S]NBMPR from

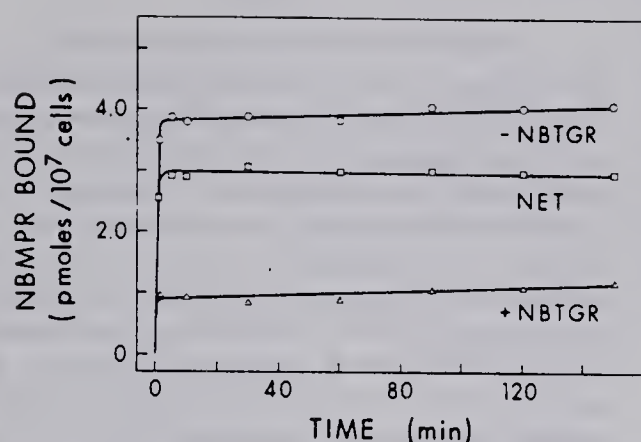


FIG. 1. Binding of NBMPR by HeLa cells at 37°

Cells from a spinner culture were resuspended in fresh medium (3.6×10^6 cells/ml) with and without $3.6 \mu\text{M}$ NBTGR. After 5 min at 37°, each culture was diluted with an equal volume of warmed medium containing [^{35}S]NBMPR (final concentration, 7.4 nM). At intervals samples were assayed for cell number and the ^{35}S content of the cell pellet. The difference between the ^{35}S content of cells incubated in the absence and presence of NBTGR (NET) was employed throughout this work to determine specifically bound NBMPR.

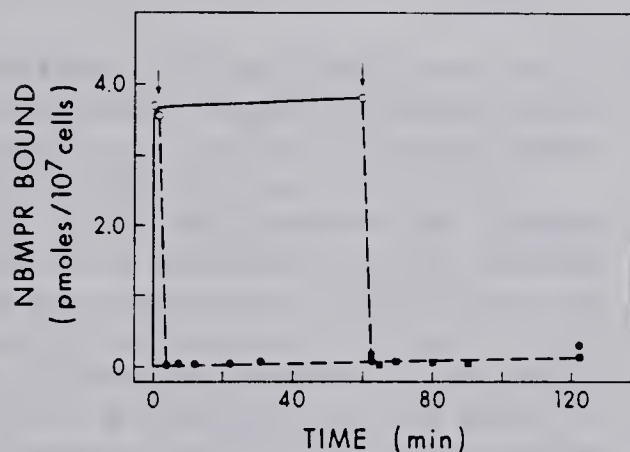


FIG. 2. Displacement of bound NBMPR from HeLa cells by NBTGR at 37°

As in Fig. 1, [^{35}S]NBMPR was added (final concentration, 6.4 nM) to replicate cell suspensions at 37°, and cell samples taken thereafter were assayed for ^{35}S . NBTGR (final concentration, $3.2 \mu\text{M}$), was added (arrow) to one group of the replicate suspensions 1.0 min (●) after addition of [^{35}S]NBMPR, and to another group (■) 59 min later (arrow); NBTGR was not added to the control group (○). Each of the replicate cell suspensions so treated had an NBTGR-treated control. To the latter, NBTGR (final concentration, $2 \mu\text{M}$) was added 5 min before the addition of [^{35}S]NBMPR, and each was processed in parallel with its untreated counterpart. Differences between the ^{35}S contents of cells with and without NBTGR treatment are plotted.

the cells by NBTGR was rapid whether latter was added early or late after contact of the cells with [^{35}S]NBMPR (Fig. 2). These data demonstrate the reversibility of NBMPR binding. Similar experiments conducted at 20° (Fig. 3) showed that association of [^{35}S]NBMPR with the binding sites was rapid (84% complete after 1 min), but displacement by NBTGR was slower at 20° than at 37° (Fig. 2). The half-life of the binding site-NBMPR complex was about 4.5 min at 20° under the displacement conditions specified in Fig. 3.

Displacement of cell-bound NBMPR by its congener, NBTGR, demonstrated reversibility of the binding. Another aspect of this reversibility is illustrated in Table 1, which shows that [^{35}S]NBMPR dissociated from cells during incubation under culture conditions in the absence of a displacing agent: successive dilutions caused the loss of successive increments of cell-bound [^{35}S]NBMPR to the medium. It is apparent in these data that NBMPR con-

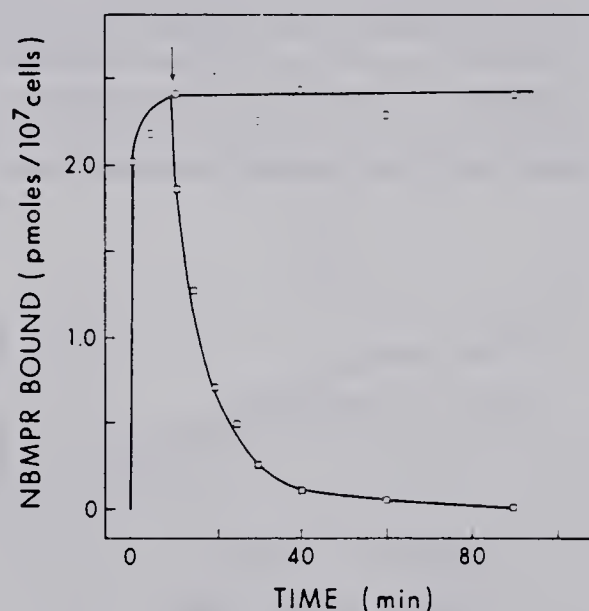


FIG. 3. Displacement of bound NBMPR from HeLa cells by NBTGR at 20°

As in Fig. 2, [^{35}S]NBMPR was added (final concentration, 5.9 nM) to replicate cell suspensions at 20°, and at various times thereafter cells were assayed for ^{35}S (○). NBTGR was added (final concentration, $2 \mu\text{M}$) 10 min (arrow) after addition of [^{35}S]NBMPR (□). Suspensions of cells pretreated with $2 \mu\text{M}$ NBTGR as in Fig. 2 were also assayed in this way. Differences between the ^{35}S contents of cells with and without NBTGR pretreatment are plotted.

TABLE 1

Dissociation of bound [35 S]NBMPR

HeLa cells were incubated at 37° in spinner medium containing [35 S]NBMPR, and the 35 S contents of pelleted cells and of medium samples were determined separately. For suspension A, cells (10^6 /ml) were incubated for 5 min. Suspension B consisted of suspension A cells resuspended in warmed, NBMPR-free medium and incubated for 60 min prior to assay of cells and medium for 35 S. For suspension C, a portion of suspension B was diluted with 3 volumes of medium, incubated for 60 min, and assayed for 35 S. For suspension D, a portion of suspension C was diluted with 2 volumes of medium, incubated for 60 min, and assayed for 35 S. NBMPR was absorbed from medium samples onto 100 mg of charcoal (grade AU-4, Barnebey-Cheney, Ltd., Columbus, Oh.), from which it was eluted with 5 ml of 1,4-dioxane (56°, 30 min) prior to assay of radioactivity.

Suspension	NBMPR in medium	Cell-bound NBMPR
	nM	pmoles/ 10^7 cells
A	4.7	1.95
B	0.12	0.41
C	0.04	0.27
D	0.02	0.26

centrations in the cell pellet were two to three orders of magnitude higher than in the medium; a portion of the cell-associated NBMPR could be intracellular.

The relationship between the site-specific binding of NBMPR and the extracellular concentration of NBMPR was explored in the experiments of Figs. 4-6. Figure 4 illustrates the basis of the binding assay; it is apparent that the ability of HeLa cells to bind NBMPR in the presence of 3.7 μ M NBTGR was greatly reduced over a wide range of NBMPR concentrations. NBTGR was added to the assay system before [35 S]NBMPR and was present at a concentration greatly in excess of free NBMPR, essentially excluding the latter from the cellular binding sites. [35 S]-NBMPR binding in the presence of NBTGR did not exceed 10% of that in its absence for concentrations of [35 S]NBMPR below 5 nM. Site-specific NBMPR binding was defined as the difference between the cellular content of 35 S in the presence and absence of NBTGR under these conditions.

Figure 5 describes the relationship be-

tween bound and free NBMPR, the latter presumably in equilibrium with the former. These data illustrate saturability of the site-specific binding of NBMPR and also show that (a) more NBMPR is bound at 37° than at 20°, and (b) binding saturation at 37° occurs at a higher concentration than at 20°.

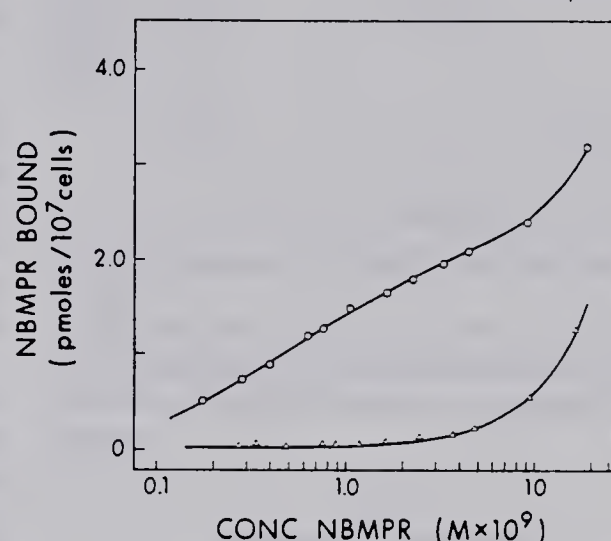


FIG. 4. Binding of NBMPR by HeLa cells

Portions (10.0 ml) of a cell suspension (1.4×10^6 cells/ml in growth medium) were mixed with 10.0 ml of calcium-free minimal essential medium containing [35 S]NBMPR with (Δ) or without (\circ) NBTGR (final concentration, 3.7 μ M) and were incubated at 37° for 5 min. The 35 S contents of cells and medium from each incubation mixture were then determined.

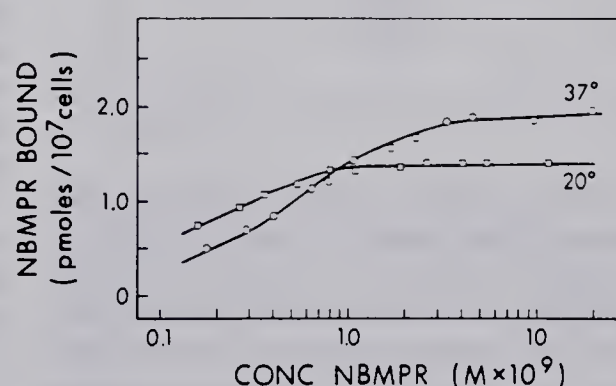


FIG. 5. Site-specific binding of NBMPR by HeLa cells at 20° and 37°

As in Fig. 4, replicate cell suspensions were incubated for 5 min at 20° (\square) or 37° (\circ) in calcium-free minimal essential medium containing [35 S]NBMPR at various concentrations with and without 3.7 μ M NBTGR. The 35 S contents of cells and medium from each suspension were measured. Specifically bound NBMPR was determined as the difference between the [35 S]NBMPR contents of cells in the presence and absence of NBTGR.

specific binding were reached at NBMPR concentrations of 0.47 nM (37°) and 0.14 nM (20°). Reciprocals of the data from Fig. 5 gave straight-line plots, from which were obtained saturation values for site-specific NBMPR binding of 2.13 (37°) and 1.48 (20°) pmoles/10⁷ cells. Site-specific binding of NBMPR at concentrations above saturation of binding in 14 independent assays averaged 2.49 ± 0.53^4 (37°) and 1.93 ± 0.38 (20°) pmoles/10⁷ cells.

Binding data from Fig. 5 were subjected to mass law analysis (Fig. 6) by the method of Scatchard (17). Because straight lines fit the data, it would appear that a single type of receptor was responsible for the site-specific binding of NBMPR by HeLa cells. These data indicate that 1.3×10^5 and 0.9×10^5 sites per HeLa cell bound NBMPR at 37° and 20°, respectively, and that NBMPR dissociation constants were 0.59 nM (37°) and 0.14 nM (20°). Table 2 summarizes results from similar experiments, which also yielded straight-line Scatchard plots at both temperatures.

To determine whether the particular NBMPR binding sites detected here were part of (or perhaps interacted with) the nucleoside transport mechanism, the ability of HeLa cells to take up thymidine (0.1 μ M) or uridine (4 μ M) was measured in the presence of graded concentrations of NBMPR. Figure 7 shows that time courses for the uptake of [*methyl*-³H]thymidine were linear for at least 2 min at 20°. The low rate of thymidine uptake in the presence of 5 μ M NBMPR is attributed to passive diffusion; other communications from this laboratory have reported that NBMPR at this concentration eliminated mediated uptake of uridine and thymidine by HeLa cells (4, 16). Use of [³⁵S]NBMPR enabled the simultaneous determination of both NBMPR binding and inhibition of nucleoside uptake. For example, when the uptake of [*methyl*-³H]thymidine was measured, $1\text{--}2 \times 10^6$ cells/assay were employed; the contribution to the observed ³H radioactivity of cell-associated [³⁵S]-NBMPR was not significant with cell samples of this size. Parallel assays with a 10-

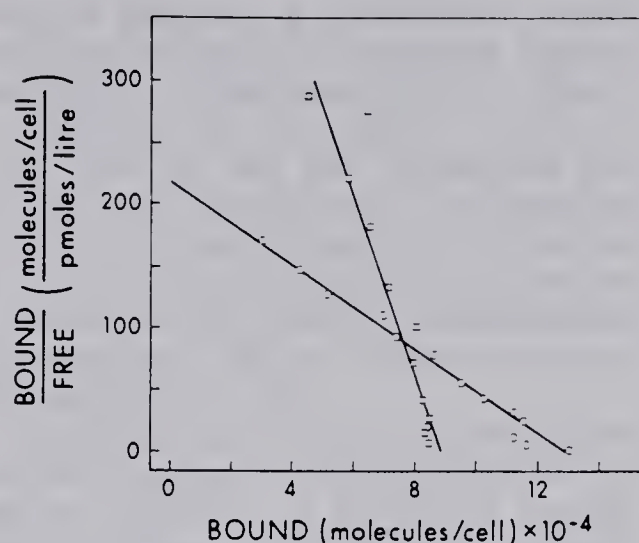


FIG. 6. Binding of NBMPR by HeLa cells at 20° and 37°

Data from the experiment of Fig. 5 are presented here in the form of a mass law (Scatchard) plot (17). Lines were fitted to the data by the method of least squares.

TABLE 2
Binding of [³⁵S]NBMPR

The site-specific binding of NBMPR to HeLa cells was measured as in Fig. 5; Scatchard plots were linear and yielded the constants listed.

Expt.	Dissociation constant		Binding sites per cell	
	20°	37°	20°	37°
	nM		$\times 10^{-5}$	
1 ^a	0.14	0.59	0.9	1.3
2		0.51		1.5
3		0.37		1.6
4	0.15		1.1	

^a Figure 6.

fold larger number of cells (and nonisotopic thymidine) enabled determination of NBMPR binding. The data of Fig. 7 show that partial occupancy of the NBMPR receptor sites resulted in partial inhibition of thymidine uptake. However, in the presence of 4.6 nM NBMPR, 2.14 pmoles of ligand were bound per 10⁷ cells, indicating nearly total occupancy of binding sites (see comments above relating to Fig. 5), yet the inhibition (75%) of thymidine uptake was well short of complete. Similar results were obtained in other experiments including that of Fig. 8. Partial occupancy of the NBMPR binding sites also resulted in partial inhibition of uridine uptake (data not shown), and, as with thymidine up-

⁴ Average deviation from the mean.

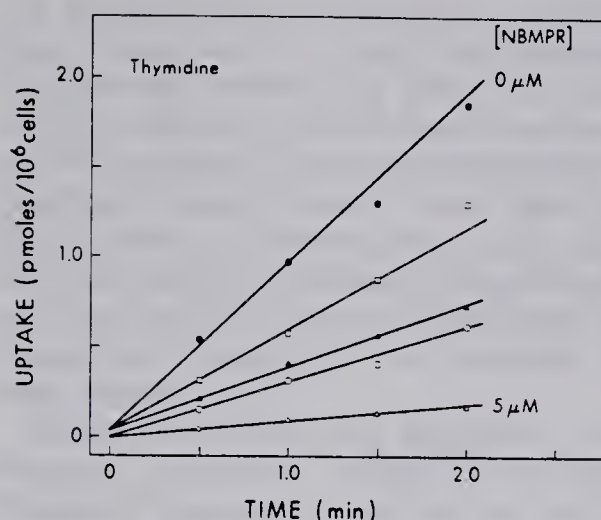


FIG. 7. Effect of bound NBMPR on uptake of thymidine by HeLa cells

The time course of thymidine uptake from medium containing $0.1 \mu\text{M}$ [*methyl- ^3H*]thymidine was assayed as described in METHODS at 20° in the absence (\bullet) and presence of [^{35}S]NBMPR (\square , \blacktriangle , \circ). Thymidine uptake was also assayed in the presence of $5 \mu\text{M}$ nonisotopic NBMPR (\triangle) to measure thymidine entry by diffusion. The binding of [^{35}S]NBMPR under conditions identical with those of the thymidine uptake assay was determined at the same time in parallel experiments which employed larger (10 -fold) cell numbers and nonisotopic thymidine. After 2 min of incubation under conditions identical with those of the thymidine uptake assay, the [^{35}S]NBMPR contents of cells and medium were determined with these results:

Free (medium) NBMPR	Bound NBMPR
nM	pmoles/ 10^7 cells
0.2 (\square)	1.10
0.7 (\blacktriangle)	1.35
4.6 (\circ)	2.14

take, as saturation values of binding were approached, uridine uptake was able to proceed at rates about 25% of control (uninhibited) values.

Thus, although different degrees of inhibitor site occupancy correlated with nucleoside transport inhibition, the relationship between these parameters was not simple. When the high-affinity sites studied here were almost fully occupied, a substantial transport capacity (25%) for uridine and thymidine remained active, yet this was inhibited when NBMPR concentrations were increased 1000 -fold to about $5 \mu\text{M}$. This result suggests the existence of NBMPR-transporter interactions distinct from those which the present binding assay detected. For example, it is possible

that the transport of a particular nucleoside may be mediated by several types of transporter, which may differ in affinity for NBMPR. In this context, it may be noted that (a) the cell populations employed were asynchronous and therefore included cells at all stages of the mitotic cycle, and (b) some nucleoside transport activities, notably those for thymidine and deoxycytidine, vary at different stages of the cell cycle (4, 18).

The experiment of Fig. 8 demonstrated that the binding of NBMPR (and the resultant inhibition of thymidine uptake) was reversible. In this experiment, cells which had bound NBMPR to the extent of

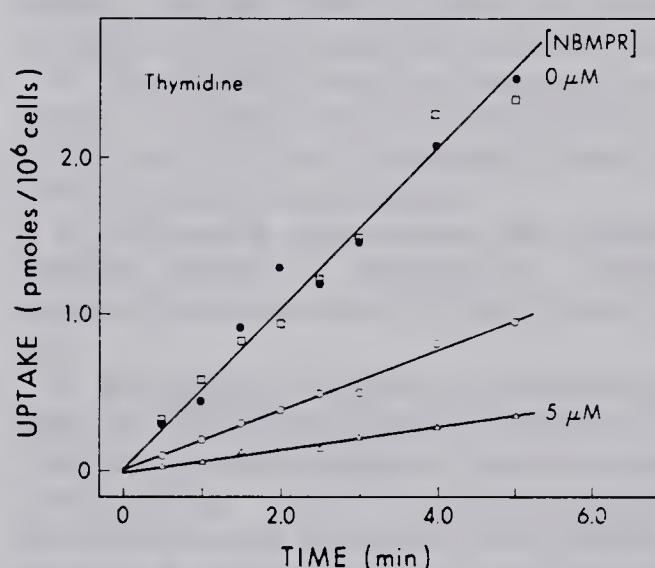


FIG. 8. Reversibility of NBMPR inhibition of thymidine uptake

Time courses of thymidine uptake by HeLa cells from medium containing $0.1 \mu\text{M}$ [*methyl- ^3H*]thymidine at 20° were determined in the absence (\bullet) and presence (\circ) of 5.5 nM [^{35}S]NBMPR. Thymidine uptake was also assayed in the presence of $5 \mu\text{M}$ nonisotopic NBMPR (\triangle) to measure the diffusional entry of thymidine. As in Fig. 7, the binding of [^{35}S]NBMPR was determined under conditions identical with those of the thymidine uptake assay in parallel experiments; in this assay, 2.2 pmoles of NBMPR were bound per 10^7 cells. The release of cell-bound NBMPR under incubation conditions at 37° was examined as follows. After incubation in the presence of 5.5 nM [^{35}S]NBMPR for 5 min at 20° , HeLa cells were resuspended in warmed growth medium (3.2×10^5 cells/ml) and incubated at 37° for 30 min. The cells were then collected by centrifugation, resuspended in growth medium (1.3×10^6 cells/ml), and assayed for uptake of [*methyl- ^3H*]thymidine (\square) as above and for their content of [^{35}S]NBMPR; the latter value was 0.21 pmol/ 10^7 cells.

2.2 pmoles/ 10^7 cells (medium concentration, 5 nM), and in which the thymidine uptake was reduced by 75%, lost bound NBMPR during a subsequent incubation at 37°; the ability to take up thymidine was regained concomitantly with this loss. This experiment demonstrated that association of NBMPR with and release from the cellular binding sites correlated with inhibition and restoration of thymidine uptake, respectively.

Identity of cell-bound NBMPR. To determine whether NBMPR underwent chemical transformation during cellular binding, HeLa cells with bound [35 S]-NBMPR were extracted with ethanol, and the extracted material was subjected to analysis by thin-layer chromatography. HeLa cell suspensions (10^6 cells/ml) were incubated for 10 min at 20° or 37° in growth medium containing 5 nM [35 S]NBMPR. Cell pellets (10^8 cells) were frozen at -76°, dried under vacuum over P_2O_5 , and then extracted with 2 ml of 70% aqueous ethanol for 10 min at 56°. The ethanolic extracts were dried, and the residues were re-extracted with absolute methanol; this procedure recovered more than 95% of the 35 S initially associated with the cell pellet. The methanolic extracts were chromatographed on paper with carrier NBMPR in three solvent systems: (a) water-saturated 1-butanol; (b) 1-butanol-glacial acetic acid-water (120:30:50, v/v/v); and (c) 95% ethanol-1 M ammonium acetate, pH 7.5 (70:30, v/v). In these systems, R_F values for NBMPR were 0.59, 0.80, and 0.93, respectively. With each solvent system, the extracted 35 S activity co-chromatographed with NBMPR, whether extracted from cells incubated at 20° or 37°. It was concluded that NBMPR remained unchanged when bound to HeLa cells.

DISCUSSION

This study has shown that HeLa cells possess high-affinity receptor sites for NBMPR. The method employed to determine site-specific binding of NBMPR measured the difference between cell-associated [35 S]NBMPR (a) under experimental conditions and (b) when the binding sites were saturated with NBTGR, a tightly

bound homologue of NBMPR (4). The following properties of the NBMPR binding sites were apparent in this study.

1. The sites bound NBMPR tightly, but reversibly. The reversible nature of the binding was apparent in the displacement of cell-bound [35 S]NBMPR by NBTGR (Figs. 1 and 2) and the dissociation of bound [35 S]NBMPR under culture conditions at 37° (Table 1 and Fig. 8). The recovery of chemically unchanged [35 S]NBMPR from cells to which it had been bound is consistent with a reversible binding process. Dissociation constants for the cell-bound NBMPR determined by mass law calculations were about 0.1 nM, indicating tight binding; dissociation constants of about 1 nM for NBMPR bound to human erythrocytes were reported previously (8).

2. The NBMPR binding sites were evidently of a single type, because of the linearity of mass law (Scatchard) plots derived from the binding data.

3. HeLa cells possess about 10^5 NBMPR binding sites; in comparison, human erythrocytes have about 10^4 such sites per cell.

4. Fractional occupancy of the binding sites by NBMPR resulted in fractional inhibition of the transport of thymidine and uridine (Figs. 7 and 8); however, the relationship between occupancy and inhibition was not a simple proportionality as with human erythrocytes (9). It would appear that interactions between the nucleoside transport mechanism and NBMPR took place (at higher concentrations of NBMPR) that were not perceived by the binding assay.

5. The maximum number of NBMPR binding sites was larger at 37° than at 20°. It is possible that this observation may have an explanation in temperature-related transitions in nucleoside uptake activity, such as those seen in Novikoff hepatoma cells (19); the latter occurred in the 15-18° range and were probably due to changes in the physical state of the membrane lipids (19).

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APPENDIX B

[CANCER RESEARCH 38, 1723-1729, June 1978]

Formation of 1- β -D-Arabinofuranosylcytosine Diphosphate Choline in Cultured Human Leukemic RPMI 6410 Cells¹

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ABSTRACT

When incubated with 1- β -D-arabinofuranosylcytosine (ara-C), RPMI 6410 cells formed a hitherto unrecognized ara-C metabolite, 1- β -D-arabinofuranosylcytosine diphosphate choline. This compound was characterized by (a) chromatographic behavior, (b) chemical and enzymatic hydrolysis, (c) phosphorus content, and (d) incorporation of [5-³H]ara-C and [methyl-¹⁴C]choline.

Formation of 1- β -D-arabinofuranosylcytosine diphosphate choline by RPMI 6410 cells was enhanced in the presence of 3-deazauridine (DU) and was preceded by that of 1- β -D-arabinofuranosylcytosine triphosphate. The antiproliferative effects of ara-C and DU toward RPMI 6410 cells were potentiated when the agents were present together. The anabolism of ara-C during a 24-hr interval of culture was markedly enhanced by the presence of DU; cellular concentrations of 1- β -D-arabinofuranosylcytosine triphosphate and 1- β -D-arabinofuranosylcytosine diphosphate choline were 5- and 15-fold higher than those in the absence of DU. This enhancement appears to be the basis of the potentiation of cytotoxicity resulting from combination of the agents.

Pretreatment of RPMI 6410 cells with DU resulted in enhanced rates of cellular uptake of ara-C. ara-C uptake under these circumstances was blocked by the inhibitor of nucleoside transport, nitrobenzylthioinosine.

INTRODUCTION

DU,⁴ a uridine analog, inhibited proliferation of microbial cells and tumor cells in culture (20) and, as a therapeutic agent, increased the survival time of mice inoculated with L1210 leukemia (2). DU was converted to 3-deazauridine triphosphate in extracts of Ehrlich ascites carcinoma cells (23) and in intact leukemia L1210 cells (13); however, incorporation of the analog into polynucleotides was not demonstrated (23). 3-Deazauridine triphosphate inhibited the activity of CTP synthetase purified from calf liver and leukemia L1210 cells in a competitive manner with a K_i of 5.3×10^{-6} M (13). The following observations indicate that DU growth-inhibitory effects result from interference with the activity of CTP synthetase: (a) growth-inhibitory effects

of DU toward microbial cells (20) and cultured cells (4, 13) were partially reversed by cytidine, uridine, and deoxycytidine but not by deoxyuridine and thymidine; and (b) pronounced depletion of cellular cytidine and deoxycytidine phosphates resulted when leukemia L1210 cells were cultured in the presence of DU (4). Brockman *et al.* (4) reported also that deazauridine diphosphate inhibited ribonucleotide reductase.

We have reported that ara-C and DU were synergistic with respect to toxicity toward mice, HeLa cells, and RPMI 6410 cells in culture (14). Resistance to the DU/ara-C combination in a line of RPMI 6410 cells deficient in uridine kinase indicated that formation of phosphorylated metabolites of DU was necessary for expression of the DU/ara-C synergism. In the presence of DU, the anabolism of ara-C in RPMI 6410 cells was greatly enhanced. After culture for 24 hr in medium containing 9 μ M ara-C and 23 μ M DU, intracellular concentrations of ara-CTP and of an unknown metabolite of ara-C were increased 3- and 8-fold, respectively, over those in cells cultured in the absence of DU (14).

In this study, the unknown ara-C metabolite was characterized as ara-CDP-choline and the kinetics of formation of this compound and of other ara-C metabolites in RPMI 6410 cells was investigated. A companion report extends these observations to other types of cells *in vivo* and *in vitro* (12).

MATERIALS AND METHODS

[5-³H]ara-C, [methyl-¹⁴C]choline, and [2-¹⁴C]ethan-1-ol-2-amine were purchased from Amersham Corp. (Oakville, Ontario, Canada). DU was generously provided by Dr. M. J. Robins, University of Alberta, and by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Nucleosides, nucleotides, 5'-nucleotidase, apyrase, phosphorylcholine, CDP-choline, CDP-ethanolamine, and dithioerythritol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cell culture materials were obtained from Grand Island Biological Co. (Calgary, Alberta, Canada). NBMPR was prepared by established methods (16) with the use of thioinosine provided by the Division of Cancer Treatment, National Cancer Institute.

RPMI 6410 cells, a myeloblastoid line derived from peripheral blood cells from an acute myeloblastic leukemia patient (15), were maintained in static culture in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum. Cell numbers, determined with an electronic particle counter, were not allowed to exceed 5×10^6 cells/ml to assure exponential growth; under these conditions they doubled every 17 to 19 hr. Every 6 to 8 weeks, cultures were restarted from *Mycoplasma*-free stocks stored in liquid nitrogen. Because of the possible

¹ Supported by the National Cancer Institute of Canada. A portion of this work was described in a preliminary communication (18).

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⁴ The abbreviations used are: DU (3-deazauridine), 4-hydroxy-1-(β -D-ribo-pentofuranosyl)-2-pyridone, ara-C, 1- β -D-arabinofuranosylcytosine, ara-CMP, ara-CDP, and ara-CTP, 5'-mono-, di-, and triphosphates of ara-C, NBMPR, 6-[[4-nitrobenzyl]thio]-9- β -D-ribofuranosylpurine; PEI, polyethyleneimine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

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presence of nucleosides in undialyzed serum, in all experiments in which RPMI 6410 cells were treated with DU or ara-C, medium containing 10% dialyzed fetal calf serum was used.

For the determination of cellular acid-soluble nucleotides, perchloric acid extracts (acid-soluble fraction) were prepared as follows. Cell suspensions were cooled to 4° and centrifuged (150 × g for 4 min at 4°); after 1 wash in cold growth medium, cell pellets were extracted at 4° with 0.4 M perchloric acid (100 µl/10⁷ cells). After reextraction of the acid-insoluble residues in the same way, the combined extracts were neutralized with KOH, freed of perchlorate, and weighed to determine volume. Cellular metabolites of [5-³H]ara-C and other labeled compounds were determined by chromatographic analysis of the neutralized acid extracts on MN300 thin layers of PEI-cellulose (Macherey-Nagel and Co., Brinkman Instruments, Westbury, N. Y.); radioactivity comigrating with appropriate markers was determined. Prior to use, impurities were washed from the PEI-cellulose sheets onto paper wicks by the ascending movement of 4.0 M ammonium formate buffer, pH 7.0; the sheets were subsequently washed in methanol and water to remove ammonium formate. Table 1 lists mobilities of various compounds on the PEI-cellulose sheets in the solvent systems used. Sections of the chromatographic medium were combusted in a Packard Model 306 Sample Oxidizer and assayed for radioactivity by a liquid scintillation method. Essentially all of the radioactivity applied to chromatograms (95 to 100%) was accounted for throughout these experiments. Ribonucleotides present in the neutralized acid extracts were determined by HPLC on a 25-cm × 4.5-mm Reeve-Angel Partisil-10SAX column (Mandel Scientific, Montreal, Quebec, Canada), with the use of a linear gradient of potassium phosphate and potassium chloride (21).

Cell volumes were obtained from cell volume distributions measured with an electronic cell counter coupled to a

Table 1
Relative mobility (R_f) on PEI-cellulose thin-layer chromatograms

Compound	R_f			
	Solvent 1 ^a	Solvent 2	Solvent 3	Solvent 4
ara-CTP	0.04	0	0	0
UTP	0.01	0	0	0
ara-CDP	0.23	0	0	0.01
UDP	0.13	0	0	
ara-CMP	0.63	0.09	0	0.05
UMP	0.39		0	
ara-C	0.91	0.58	0.22	0.82
ara-U ^b	0.86		0.58	
Uridine	0.86		0.43	
ara-CDP-choline	0.79	0.41	0	0.60
CDP-choline	0.79	0.41	0	0.23
CDP-ethanolamine	0.77	0.34	0	0.15
Phosphorylcholine		0.63		

^a Solvent 1: the following solvents were used sequentially without drying between changes; the front was run to 2 cm above the origin with 1 N acetic acid, then to 8 cm with 0.66 N acetic acid containing 0.33 M LiCl, and finally to 16 cm with 0.66 N acetic acid containing 0.66 M LiCl. Solvent 2: 0.1 N acetic acid. Solvent 3: ethyl acetate:isopropyl alcohol:water (65:22.5:12.5, v/v). Solvent 4: 0.1 M ammonium formate containing 2% boric acid (pH 7.4).

^b ara-U, 1-β-D-arabinofuranosyluracil.

multichannel particle size analyzer (Coulter Electronics, Hialeah, Fla.) calibrated with polystyrene microspheres (Coulter).

Among the criteria used in the characterization of particular nucleotide products was susceptibility to hydrolysis by snake venom 5'-nucleotidase and potato apyrase. 5'-Nucleotidase reaction mixtures contained, in a total volume of 600 µl, 50 mM Tris-glycine buffer (pH 9.0), 10 mM MgCl₂, 0.1% mercaptoethanol, 1.5 units of 5'-nucleotidase, and the test substrate. Reactions were allowed to proceed for 30 min at 37° and then stopped by heating at 100° for 3 min; reaction mixtures were clarified by centrifugation (8000 × g for 1 min), freeze-dried, and analyzed by chromatography. Apyrase reaction mixtures contained 100 mM potassium succinate buffer (pH 6.5), 1.8 mM CaCl₂ and, in a final volume of 100 µl, 1 unit of apyrase and the test substrate. Reactions proceeded for 30 min at 37°, were stopped by heating at 100° for 3 min, and were analyzed by chromatography.

Kennedy's method for synthesis of CDP-choline (9) was used for the preparation of ara-CDP-choline. The product was isolated by anion-exchange chromatography on a 0.9 × 15-cm column of Dowex 1-formate with elution by a linear gradient changing from water to 0.05 N formic acid in 600 ml (Chart 2); CDP-choline and ara-CDP-choline eluted together. Yields of ara-CDP-choline (about 3.5%) were lower than those reported for CDP-choline synthesis (9). The UV absorption spectrum (pH 2) of the ara-CDP-choline product was identical with that of CMP. The molar ratio of the phosphate content (1) of the product to that of cytosine was 2.05. At pH 2 and 280 nm (λ_{max}), the molar extinction coefficient of ara-CDP-choline was 13.6×10^3 , calculated on the basis of 2 atoms of phosphorus per mol. Chromatographic mobilities of ara-CDP-choline and CDP-choline were identical in Systems 1 to 3 (Table 1), but the former migrated more rapidly in System 4. Following acid hydrolysis of the synthetic product (1 N H₂SO₄ or HCl, 1 hr at 100°), UV-absorbing material migrated with authentic ara-CMP in Systems 1 and 2. After treatment of the product with apyrase, UV-absorbing material migrated with ara-C in Systems 1 and 3; this result was consistent with the apparent identity of the product because the apyrase used was known to contain nucleotide monophosphatase activity. Together, these results established the identity of the synthetic product as ara-CDP-choline. Attempts to prepare ara-CDP-ethanolamine by this procedure have not been successful.

Rates of cellular uptake of ara-C were assayed as follows. Cell suspensions in RPMI 1640 medium with 5% dialyzed fetal calf serum were used; intervals of ara-C uptake were initiated by additions of [5-³H]ara-C to achieve a final concentration of 5 µM. Intervals of uptake were terminated by transferring 1.0-ml samples of the assay mixtures into 40 ml of cold 0.154 M NaCl; after centrifugation (500 × g for 3 min at 4°), cell pellets were washed once with 20 ml of cold 0.154 M NaCl and dissolved in 0.3 ml of NCS tissue solubilizer (Amersham) for assay of ³H by liquid scintillation counting with Bray's fluor solution (3). Deoxycytidine kinase activity was measured by the method of Cheng and Ostrander (6), and protein was determined by the method of Hartree (7).

RESULTS

In an earlier report, we showed that the growth-inhibitory effects of ara-C and DU toward RPMI 6410 cells were enhanced synergistically when these agents were present together (14); in this study, the surviving fractions of cells treated for 24 hr with both agents ($9 \mu\text{M}$ DU and $3 \mu\text{M}$ ara-C) were approximately one-tenth of that expected from the sum of the cytotoxic effects of the separate agents. Table 2 lists concentrations of soluble nucleotides in RPMI 6410 cells following 24 hr of culture in the presence of ara-C, DU, or a combination of both. The pronounced depletion of cellular concentrations of cytidine phosphates in the presence of DU agrees with an earlier report by Brockman *et al.* (4).

In the experiment of Chart 1, RPMI 6410 cells were cultured for 24 hr in the presence of $[5\text{-}^3\text{H}]\text{ara-C}$ and DU, and the cellular acid-soluble fraction was analyzed by TLC for metabolites of ara-C. About 60% of the cellular ^3H activity was in the form of an unknown metabolite that comigrated with synthetic ara-CDP-choline, and 25% of the applied radioactivity accompanied ara-CTP. The unknown metabolite, subsequently characterized as ara-CDP-choline, accounted for a major portion of the ara-C anabolites formed in the presence of DU. The cellular content of ara-CTP and ara-CDP-choline increased approximately 5- and 15-fold, respectively, when DU was present (Table 3). ara-C-derived ^3H activity comigrated with CDP-ethanolamine and was presumed to indicate formation of ara-CDP-ethanolamine (Table 3); the incorporation of $[2\text{-}^{14}\text{C}]\text{ethanolamine}$ into this compound (see below) supported this supposition. The putative ara-CDP-ethanolamine was consistently a minor ara-C metabolite, relative to ara-CDP-choline.

Characterization of the Unknown ara-C Metabolite as ara-CDP-choline

Preparative Isolation of the Presumed $[^3\text{H}]\text{ara-CDP-choline}$. A number of cultures of RPMI 6410 cells were incubated for 24 hr in medium containing $3 \mu\text{M}$ $[5\text{-}^3\text{H}]\text{ara-C}$ (360 cpm/pmol) and $9 \mu\text{M}$ DU; neutralized acid-soluble fractions from these cultures, representing 4.2×10^7 cells, were

Table 2

Nucleotide content of RPMI 6410 cells

Following 24 hr of incubation in medium containing $3 \mu\text{M}$ ara-C, $9 \mu\text{M}$ DU, or both, cultures were chilled, cells were collected, and perchloric acid extracts thereof were analyzed by HPLC. The increases in nucleotide content of cells after 24 hr of culture in medium containing DU and/or ara-C are related evidently to the approximate doubling in cell volume that occurred during the 24-hr interval under these circumstances.

Nucleotide	Nucleotide concentration (nmol/ 10^6 cells) in cells after incubation in medium containing these agents			
	Control	ara-C	DU	ara-C + DU
GTP	883	1873	1541	1421
ATP	3213	6939	5789	5043
CTP	249	528	0	66
UTP	1029	1954	1735	1081
ADP	411	649	567	427
UDP	108	44	146	0
CDP	122	112	0	0
GDP	53	65	28	48

ara-CDP-Choline in Human Leukemic Cells

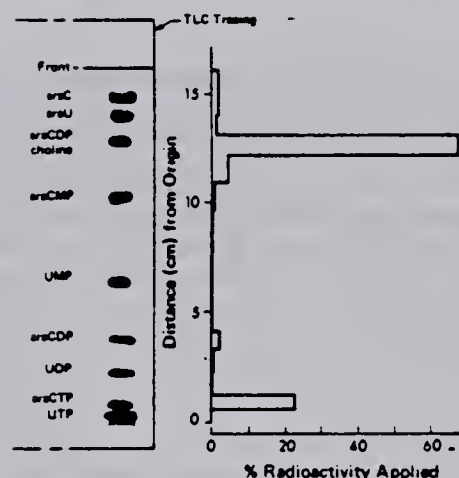


Chart 1. Metabolites of ara-C formed by RPMI 6410 cells in the presence of DU. Cells were cultured in medium containing $3 \mu\text{M}$ $[5\text{-}^3\text{H}]\text{ara-C}$ and $9 \mu\text{M}$ DU for 24 hr. Perchloric acid extracts of the cells were chromatographed on a thin layer of PEI-cellulose with the use of Solvent 1. araU, 1- β -D-arabinofuranosyluracil.

Table 3

Metabolites of ara-C in RPMI 6410 cells

Cells were cultured for 24 hr in medium containing $3 \mu\text{M}$ $[5\text{-}^3\text{H}]\text{ara-C}$ with and without $9 \mu\text{M}$ DU; perchloric acid extracts of the cells were analyzed by TLC with Solvent 1.

Metabolite	Cellular concentration (nmol/ 10^6 cells)	
	- DU	+ DU
ara-CTP	7.6	39.0
ara-CDP	0.3	3.1
ara-CMP	0	0.9
ara-CDP-choline	8.8	135.3
ara-CDP-ethanolamine	1.1	10.90
ara-C	1.8	3.3

pooled and lyophilized. The combined material was applied as a 15-cm streak on a PEI-cellulose thin-layer chromatogram that was developed in Solvent 2. The chromatographic medium adjacent to ara-CDP-choline marker spots was extracted at 4° with 0.5 N acetic acid and concentrated by freeze-drying. The ^3H activity of this preparation comigrated with ara-CDP-choline on PEI-cellulose thin layers in Solvents 1, 2, and 4; the procedure yielded 1.7 nmol (ara-C equivalent) of the metabolite. Chart 2 shows that the ara-C metabolite and authentic ara-CDP-choline had similar chromatographic mobilities on Dowex 1-formate resin.

Hydrolysis of the ara-C Metabolite. ara-CMP and phosphorylcholine are products expected from the acid hydrolysis of ara-CDP-choline (9); in accordance with this, when the $[^3\text{H}]\text{ara-C}$ metabolite (320 pmol) was heated in 1 N sulfuric acid for 1 hr at 100° , $[^3\text{H}]\text{ara-CMP}$ was formed (Table 4). When the hydrolysate was treated with snake venom $5'$ -nucleotidase, better than 90% of the ^3H activity comigrated with ara-C on PEI-cellulose thin layers in Solvents 1 and 2, indicating that the acid hydrolysis product was a nucleoside $5'$ -phosphate. Without acid hydrolysis, treatment with $5'$ -nucleotidase did not affect the chromatographic behavior of the ara-C metabolite.

Following treatment of the ara-C metabolite with apyrase, most of the ^3H cochromatographed with ara-C (Table 4);

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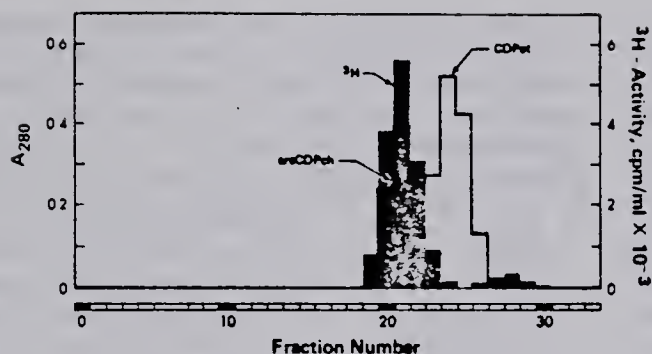


Chart 2. Chromatographic behavior of the unknown ara-C metabolite. The metabolite of $[5\text{-}^3\text{H}]\text{ara-C}$, presumed to be ara-CDP-choline (araCDPch; isolated by TLC from RPMI 6410 cells as described in the text), was applied at pH 8.5 to a 0.9- x 15-cm column of Dowex 1-formate along with 0.5 μmol each of ara-CDP-choline and CDP-ethanolamine (CDPet). The column was washed with 200 ml water and eluted with a gradient of formic acid that was linear between 0 and 0.05 M (600 ml). The flow rate was 0.55 ml/min, and 5.5-ml fractions were collected.

Table 4
Hydrolysis of "ara-CDP-choline"

The substance presumed to be $[^3\text{H}]\text{ara-CDP-choline}$ (isolated from cells incubated with $[5\text{-}^3\text{H}]\text{ara-C}$) was treated with apyrase or with 1 N sulfuric acid (100° , 1 hr), and samples of the reaction mixtures were analyzed as described in Table 3.

Chromatogram marker	Distribution of ^3H after these treatments (% radioactivity applied)		
	None	Apyrase	Acid hydrolysis
ara-C	0	73	1
ara-CMP	0	5	93
ara-CDP-choline	98	20	4

because the apyrase preparation was known to contain nucleotide phosphomonoesterase activity, this result indicated that the apyrase cleavage of a pyrophosphate linkage was followed by a dephosphorylation of the apparent product, ara-CMP. Similar results were obtained with authentic ara-CDP-choline.

Choline as a Constituent of the ara-C Metabolite. The chromatographic behavior of the ara-C metabolite suggested identity with ara-CDP-choline, as did its hydrolysis products; this idea was tested by determining whether $[\text{methyl-}^{14}\text{C}]\text{choline}$ was incorporated into the metabolite. In order to minimize the possibility of a simultaneous incorporation of ^{14}C from this source into CDP-choline, in this experiment (Chart 3) we used cells depleted of cytidine phosphates by prior treatment with DU. Preliminary experiments showed that incubation for 6 hr in growth medium containing 9 μM DU reduced concentrations of cytidine phosphates in RPMI 6410 cells to essentially zero, as determined by HPLC analysis of cell extracts; cells were not depleted of other ribonucleotides under this condition. In the experiment of Chart 3, a culture of RPMI 6410 cells was pretreated with DU as above and divided; $[5\text{-}^3\text{H}]\text{ara-C}$ and $[\text{methyl-}^{14}\text{C}]\text{choline}$ were added to one portion (Suspension A), and $[\text{methyl-}^{14}\text{C}]\text{choline}$ was added to the other (Suspension B). Incubation at 37° was then continued for 18 hr. Perchloric acid extracts of the cells from both suspensions were subjected to the Dowex 50- H^+ chromatographic procedure of Chart 3, which separated ara-CDP-choline from

choline and phosphorylcholine; ara-CDP-choline and CDP-choline eluted together in this system. Chart 3 shows that ^3H and ^{14}C from Suspension A cells eluted together and with ara-CDP-choline, suggesting that both precursors were incorporated into the putative ara-CDP-choline. Since ^{14}C activity from Suspension B cells did not elute at the ara-CDP-choline position (Chart 3), it was concluded that CDP-choline was not formed; apparently, the DU-induced depletion of cellular pools of cytidine phosphates precluded formation of CDP-choline. Thus, under the conditions of this experiment, the presence of ara-C was necessary for the incorporation of $[\text{methyl-}^{14}\text{C}]\text{choline}$ into the putative ara-CDP-choline.

When Fractions 5 to 7 from the Suspension A eluate (Chart 3) were pooled, freeze-dried, and subjected to TLC on PEI-cellulose in Solvents 1 and 4, the ^3H and ^{14}C present comigrated together with ara-CDP-choline. On chromatograms developed in Solvent 4, which separated CDP-choline and ara-CDP-choline, ^{14}C activity did not accompany the CDP-choline marker, indicating that synthesis of CDP-choline did not occur during the 18-hr incubation. When the doubly labeled ara-CDP-choline (Chart 3A) was hydrolyzed (1 N HCl, 100° for 1 hr) and subjected to TLC analysis with the use of Solvent 2, ^3H and ^{14}C activities migrated with ara-CMP and phosphorylcholine, respectively (data not shown).

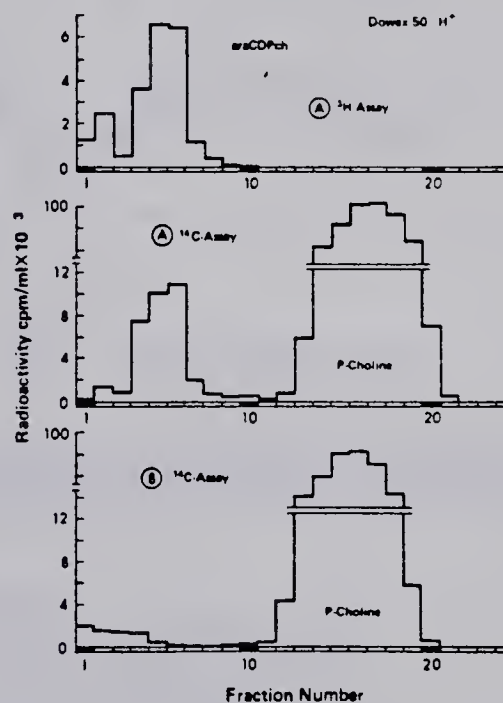


Chart 3. Incorporation of $[\text{methyl-}^{14}\text{C}]\text{choline}$ into the unknown ara-C metabolite. RPMI 6410 cells were incubated for 6 hr in Medium 199 containing 9 μM DU and, without change of medium, the culture was divided. To one portion (A), $[5\text{-}^3\text{H}]\text{ara-C}$ and $[\text{methyl-}^{14}\text{C}]\text{choline}$ were added, the former at 3 μM ; addition of the latter represented an increment of 19.2 μM (1.8×10^6 cpm/ml) to the choline content of the medium. To the other portion (B), only $[\text{methyl-}^{14}\text{C}]\text{choline}$ was added, as in A. After a further 18 hr, neutralized perchloric acid extracts of the cells were prepared, and portions corresponding to 10^7 cells were applied to 0.5- x 5-cm Dowex 50- H^+ columns that were eluted with water; 1-ml fractions were collected and assayed for ^3H and ^{14}C activities by the oxidation-liquid scintillation method. araCDPch, ara-CDP-choline; P-choline, phosphorylcholine.

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Isolation of *ara*-CDP-choline. Neutralized perchloric acid extracts of the cells were accumulated from a series of RPMI 6410 cell cultures incubated for 24 hr in the presence of 3 μ M *ara*-C and 9 μ M DU, and a pool of such extracts representing 1.45×10^9 cells was chromatographed on Dowex 1-formate (see Chart 2) together with [3 H]*ara*-CDP-choline to serve as a marker. Eluate Fractions 24 to 28, which contained *ara*-CDP-choline, were freeze-dried and rechromatographed on Dowex 50-H⁺ as shown in Chart 3. Pooled eluate fractions that represented 44% of the marker 3 H were freeze-dried. The UV absorption spectrum of the product thus obtained was identical with that of CMP, and the molar ratio of the phosphorus content (1) to that of cytosine was 2.0; when subjected to TLC on PEI-cellulose with Solvents 1 and 4, the product comigrated with *ara*-CDP-choline.

***ara*-C Metabolism in RPMI 6410 Cells**

***ara*-CDP-ethanolamine as a Metabolite of *ara*-C.** In an experiment similar to that of Chart 3, RPMI 6410 cells were pretreated for 6 hr with 9 μ M DU and then incubated with (a) [3 H]*ara*-C (3 μ M) and [14 C]ethanolamine (9.1 μ M), or (b) [14 C]ethanolamine alone for 18 hr. Acid-soluble extracts from such cells were chromatographed on Dowex 50-H⁺ columns (as in Chart 3), and fractions that eluted with CDP-ethanolamine were freeze-dried and analyzed by PEI-cellulose TLC in Solvent 1. 3 H and 14 C in such fractions from cells incubated with both *ara*-C and ethanolamine comigrated with CDP-ethanolamine. No 14 C was associated with CDP-ethanolamine in extracts from cells incubated with [14 C]ethanolamine in the absence of *ara*-C. These observations indicate that *ara*-CDP-ethanolamine is a metabolite of *ara*-C in RPMI 6410 cells.

Formation of *ara*-CTP and *ara*-CDP-choline. The effect of DU on the time courses of *ara*-CTP and *ara*-CDP-choline formation in RPMI 6410 cells is apparent in the data of Chart 4. In cells cultured in the presence of 9 μ M DU, *ara*-C anabolism was enhanced and cellular concentrations of *ara*-CTP and *ara*-CDP-choline were maximal after 14 hr of incubation. Formation of *ara*-CTP clearly preceded that of *ara*-CDP-choline, a finding consistent with formation of *ara*-CDP-choline by the phosphorylcholine cytidyltransferase (EC 2.7.7.15) reaction. In the absence of DU, cellular concentrations of *ara*-CTP peaked within 3 hr of incubation and fell thereafter; the cellular content of *ara*-CDP-choline in-

creased gradually, until after 22 hr of incubation cellular levels of both metabolites were similar.

Table 5 shows that the DU enhancement of *ara*-C anabolism was essentially abolished in the presence of deoxycytidine and markedly reduced by cytidine and uridine. In the presence of cytidine, the ratio of *ara*-CTP to *ara*-CDP-choline was markedly increased, possibly suggesting competition between CTP and *ara*-CTP for phosphorylcholine cytidyltransferase.

Effect of DU Pretreatment on *ara*-C Uptake. The influence of the duration of DU exposure on the ability of the myeloblastoid cells to take up *ara*-C was examined in the experiment of Chart 5; these data show that initial rates of *ara*-C uptake increased with the time of DU exposure. Cells increased in volume during exposure to DU; however, increases in *ara*-C uptake rate were not directly related to the volume increases, although the latter may have contributed to the former to the some extent. Culture for 12 hr in medium containing 9 μ M DU did not change cell volume (mean and modal volumes were about 30 and 23 cu μ m, respectively, in the presence or absence of DU); however, uptake rates of *ara*-C were increased about 7-fold by this

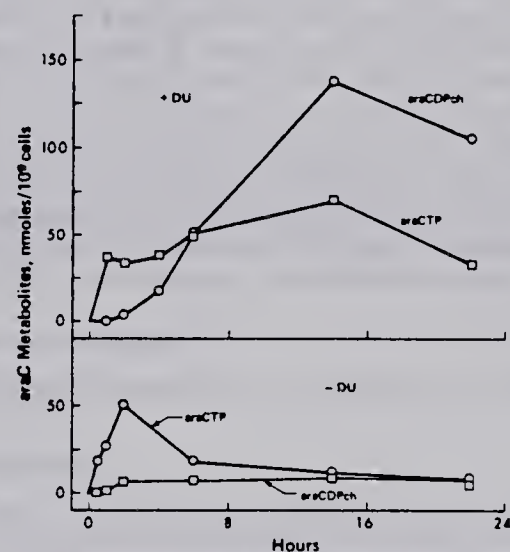


Chart 4. Time course of *ara*-CTP and *ara*-CDP-choline (*ara*CDPch) formation. RPMI 6410 cells were cultured in medium containing 3 μ M [3 H]*ara*-C with and without 9 μ M DU; at the indicated intervals, cell extracts were analyzed as described in Chart 1.

Table 5
Effect of nucleosides on anabolism of *ara*-C

RPMI 6410 cells were cultured for 24 hr in medium containing 3 μ M [3 H]*ara*-C, 9 μ M DU, and the indicated nucleosides (10 μ M). Perchloric extracts of cells were analyzed as described in Table 3.

Nucleoside	Total <i>ara</i> -C metabolites (nmol/10 ⁹ cells)	Distribution of radioactivity (% of total)					
		<i>ara</i> -CTP	<i>ara</i> -CDP	<i>ara</i> -CMP	<i>ara</i> -CDP-choline	<i>ara</i> -CDP-ethanolamine	<i>ara</i> -C
None	143	36.2	2.1	0.4	44.6	13.0	3.6
Deoxycytidine	6.5	29.5	1.5	0.5	26.8	22.2	4.2
Cytidine	24.5	71.3	3.0	0.6	13.3	8.0	4.4
Uridine	65.6	58.3	2.1	0.4	20.3	12.2	6.7
Thymidine	139	41.7	1.8	0.2	41.3	10.9	3.9

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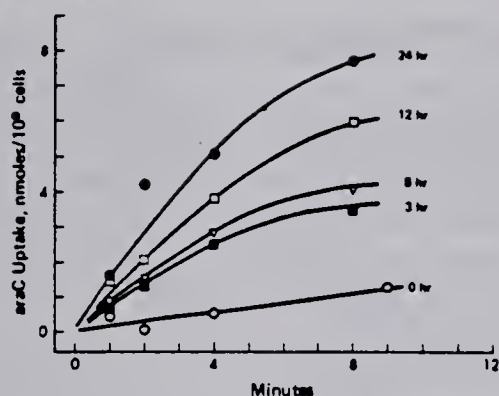


Chart 5. Increase of ara-C uptake rate by RPMI 6410 cells with increasing interval of DU exposure. After culture in medium containing $9 \mu\text{M}$ DU for the intervals indicated, cells were washed in warmed, DU-free medium and assayed for their ability to take up ara-C from medium containing $5 \mu\text{M}$ [^3H]ara-C and 5% dialyzed fetal calf serum. Two confirmatory experiments gave similar results.

interval of exposure to DU (Chart 5). Culture for 24 hr in medium containing $9 \mu\text{M}$ DU doubled the mean cell volume and increased the rate of cellular uptake of ara-C 8-fold, relative to cells without DU exposure. Thus, the ara-C uptake rate varied independently of cell volume. The basis of the DU-induced increase in ara-C uptake is not known, but it may be related to the depletion of cellular cytosine nucleotides that occurs in the presence of DU.

The DU-enhanced uptake of ara-C, assayed as described in Chart 5, was sensitive to inhibition by $5 \mu\text{M}$ NBMPR (data not shown), a potent inhibitor of nucleoside transport (17, 19). Since NBMPR at a similar concentration did not affect phosphorylation of [^3H]ara-C by homogenates of RPMI 6410 cells (data not shown), it may be concluded that entry of ara-C into the intact cells is mediated by a transport mechanism. This idea was supported by demonstrating that RPMI 6410 cells were "protected" against growth-inhibitory effects of ara-C and DU by the presence of $5 \mu\text{M}$ NBMPR (data not shown). Evidence has been presented previously that ara-C is a substrate for the nucleoside transport mechanism of human erythrocytes (5).

DISCUSSION

The formation of CDP-choline is catalyzed by phosphorylcholine cytidyltransferase (11):



This enzyme also accepts dCTP as a substrate (10) and the product, dCDP-choline, has been demonstrated in various tissues (10, 22). The present study indicates that ara-CTP will also serve as substrate in this reaction. Both CDP-choline (11) and dCDP-choline (10) serve as donors of the phosphorylcholine moiety in phosphatidylcholine synthesis; whether ara-CDP-choline will also participate in this reaction is not known. The appearance of ara-CTP as a metabolite of ara-C in RPMI 6410 cells preceded that of ara-CDP-choline, as would be expected from the above scheme.

This study has established ara-CDP-choline as a major metabolite of ara-C in RPMI 6410 cells; this compound has

not been described previously or reported as an ara-C metabolite. It is not known whether formation of ara-CDP-choline in cells contributes to ara-C cytotoxicity, either in the general sense or in particular cell types such as RPMI 6410 cells. In view of the reversibility of the phosphorylcholine cytidyltransferase reaction (11), it is possible that the intracellular pool of ara-CDP-choline may sustain that of ara-CTP.

Under the circumstances of the DU enhancement of ara-C anabolism, the principal ara-C metabolites were ara-CTP and ara-CDP-choline. It would appear that the enhancement of ara-C anabolism is (a) an effect of the DU-induced reduction in the cellular content of cytidine phosphates (and also, presumably, of deoxycytidine phosphates) and (b) the basis of the synergistic enhancement of cytotoxicity that resulted when RPMI 6410 cells were treated with ara-C and DU together.

Pretreatment of the myeloblastoid cells with DU enhanced initial rates of cellular ara-C uptake. Because the nucleoside transport inhibitor, NBMPR, blocked ara-C uptake under these circumstances, a transport step is evidently part of the ara-C uptake process. It is possible that the DU enhancement of ara-C uptake is the result of activity changes (such as release from allosteric inhibition) in 1 or more of the steps comprising the ara-C uptake process, perhaps transport or phosphorylation steps. We suggest that changes in the activity of deoxycytidine kinase may be involved in the enhancement because (a) DU profoundly reduced cellular concentrations of cytidine phosphates (Table 2) and, presumably, also those of the deoxycytidine phosphates, as reported by Brockman et al. (4), (b) dCTP is an allosteric inhibitor of deoxycytidine kinase activity (8), and (c) the DU enhancement of ara-C metabolism was abolished in the presence of deoxycytidine (Table 5).

ACKNOWLEDGMENTS

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APPENDIX C

[CANCER RESEARCH 38, 1730-1733, June 1978]

Formation of 1- β -D-Arabinofuranosylcytosine Diphosphate Choline in Neoplastic and Normal Cells¹

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ABSTRACT

1- β -D-Arabinofuranosylcytosine diphosphate choline was formed from 1- β -D-arabinofuranosylcytosine (ara-C) during incubation *in vitro* of peripheral myeloblasts from patients with acute myelogenous leukemia and cultured cells (nonleukemic human lymphocytes, mouse lymphoma L5178Y, and HeLa); as well, 1- β -D-arabinofuranosylcytosine diphosphate choline was formed *in vivo* in mouse leukemia L1210 cells and mouse liver. 3-Deazauridine enhanced the anabolism of ara-C in nonleukemic lymphocytes *in vitro* and leukemia L1210 cells *in vivo* but did not influence ara-C anabolism in the other cell types. In acute myelogenous leukemia myeloblasts incubated *in vitro* with ara-C, concentrations of 1- β -D-arabinofuranosylcytosine 5'-triphosphate were maximal after 8 hr of incubation, and formation of the latter preceded that of 1- β -D-arabinofuranosylcytosine diphosphate choline.

INTRODUCTION

The accompanying report (4) demonstrates that (a) ara-CTP⁴ and ara-CDP-choline are major metabolites of ara-C in RPMI 6410 cells, a line of cultured human myeloblastoid cells; (b) the anabolism of ara-C in these cells was enhanced in the presence of DU; and (c) the cytotoxicity of ara-C and DU was enhanced synergistically when the cells were exposed to these agents in combination. In this report, observations on the metabolism of ara-C and the influence of DU on this process are extended to include other types of cells.

MATERIALS AND METHODS

Analytical methods and procurement of materials were as described in the preceding report (4) unless specified otherwise. The identity of ara-C metabolites listed in this study was confirmed in at least 2 of the solvent systems specified in the preceding report (4).

HeLa cells were cultured as described previously (8). A cloned line of the L5178Y lymphoma was used (9); cultures were restarted at frequent intervals from frozen stocks and were maintained in static culture in Fischer's medium

containing 10% horse serum at 37° in 5% CO₂-air. Cell numbers were kept below 5×10^6 cells/ml, and doubling times were about 11 hr. LS cells, a line of human, nonleukemic lymphocytes obtained through the courtesy of Dr. J. E. Seegmiller, University of California, San Diego, Calif., were maintained in static culture in Roswell Park Memorial Institute Medium 1640 containing 20% fetal calf serum at 37° in 5% CO₂-air. When cell proliferation was exponential, the population-doubling time was about 30 hr. Dialyzed sera were used when the metabolism of ara-C was studied in these different types of cells. For the collection of peripheral myeloblasts from AML patients, blood samples were mixed with 0.25 volume of Plasmagel (Roger Bellon Laboratories, Neuilly, France) and left undisturbed for 40 min at 21° to allow cell aggregates to settle. The unsettled upper portion of the suspension containing the myeloblasts was centrifuged ($200 \times g$ for 5 min), and the cells thus obtained were washed once with 15 ml of warmed Roswell Park Memorial Institute Medium 1640 containing 10% dialyzed fetal calf serum and then incubated at 37° in the same medium with drugs as indicated below. Cell numbers were determined with an electronic particle counter (Coulter Electronics Ltd., Hialeah, Fla.).

Metabolites of ara-C in leukemia L1210 cells *in vivo* were determined as follows. Six days after i.p. implantation with 10^6 L1210 cells, female C57BL \times DBA/2 F₁ (hereafter called BDF₁) mice received i.p. injections (0.2 ml) of DU or [5-³H]ara-C in 0.154 M NaCl in dosages and sequences given below. After such treatment, ascitic fluid from each mouse was collected separately in 50 ml of 0.154 M NaCl at 4°, and the leukemic cells (3 to 5×10^6) were recovered by centrifugation ($200 \times g$, 5 min at 4°) and washed once. Perchloric acid extracts of the cell pellets were analyzed by thin-layer chromatography (4).

Metabolites of ara-C in liver samples from BDF₁ mice treated i.p. with [5-³H]ara-C and DU were determined in the following manner. At intervals after the injection of drugs, mice were killed by cervical dislocation; within 5 sec a portion of liver (about 100 mg) was frozen between metal clamps cooled in liquid nitrogen. The liver samples were pulverized while still frozen with 0.3 ml of 0.4 M HClO₄ in a liquid nitrogen-chilled container with the use of a Mikro-Dismembrator (B. Braun, Melsungen, West Germany) at full amplitude for 30 sec. The extracts were thawed to 4°, left for 15 min on ice, and centrifuged at $8000 \times g$ for 1 min (Eppendorf Model 3200 microcentrifuge). The residues were reextracted with 0.1 ml of cold 0.4 M HClO₄, and the extracts were combined, neutralized, weighed to determine volume, and then analyzed by thin-layer chromatography (4). The protein content of the acid-insoluble residues was determined by the method of Hartree (2).

¹ Supported by the National Cancer Institute of Canada. A portion of this work was described in a preliminary communication (5).

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⁴ The abbreviations used are: ara-CMP, ara-CDP, and ara-CTP, the 5'-mono-, di-, and triphosphates of 1- β -D-arabinofuranosylcytosine; ara-C, 1- β -D-arabinofuranosylcytosine; DU (3-deazauridine), 4-hydroxy-1-(β -D-ribofuranosyl)-2-pyridone; AML, acute myelogenous leukemia.

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RESULTS AND DISCUSSION

The aim of this study was to determine whether ara-C metabolites identified in RPMI 6410 cells (4), notably ara-CDP-choline and ara-CDP-ethanolamine, were also formed in other types of cells and whether the anabolism of ara-C in the latter was influenced by DU. Myeloblasts from AML patients were incubated with [5-³H]ara-C in the presence or absence of DU. It is evident from Chart 1 that the formation of ara-CTP and ara-CDP-choline in myeloblasts from Patient W. L. was not influenced by the presence of DU. In the presence of 3 μ M ara-C, cellular concentrations of ara-CTP were maximal after 8 hr and fell thereafter; those of ara-

CDP-choline reached a plateau after about 8 hr. The formation of ara-CTP preceded that of ara-CDP-choline, a finding consistent with the route by which ara-CDP-choline is synthesized in cells (4). When the myeloblasts were incubated with 0.3 μ M [5-³H]ara-C, concentrations of ara-CTP were also maximal after 8 hr; those of ara-CDP-choline increased linearly for 24 hr. Table 1 lists nucleotide concentrations in the same myeloblasts after 24 hr of culture in the presence or absence of 9 μ M DU. The ATP content of the myeloblasts was maintained during the 24-hr interval of culture. It is also evident that the presence of DU did not affect the CTP content of the myeloblasts, in contrast to the situation with RPMI 6410 cells (4) and leukemia L1210 cells (1).

The data of Table 2 demonstrate the formation of ara-C metabolites in myeloblasts from 2 other AML patients: ara-CDP-choline was a major metabolite of ara-C, accounting for 8 to 30% of the total acid-soluble radioactivity. With myeloblasts from Patient M. W., DU had little effect on the total anabolism of ara-C; however, the proportion of the radioactivity associated with 1- β -D-arabinofuranosyluracil 5'-monophosphate (included in 1- β -D-arabinofuranosyluracil phosphates in Table 2) was reduced considerably in the

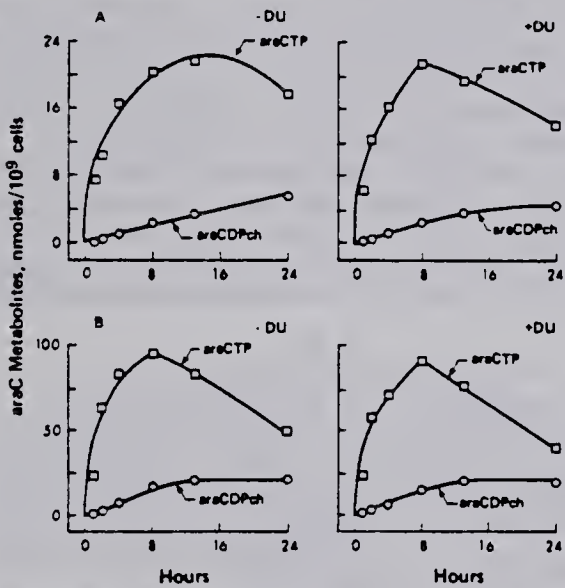


Chart 1. Time course of ara-CTP and ara-CDP-choline (araCDPch) formation in AML myeloblasts. Myeloblasts from Patient W. L. were incubated in medium containing 0.3 μ M (A) or 3 μ M (B) [5-³H]ara-C with or without 9 μ M DU. At the indicated intervals, cell extracts were analyzed by thin-layer chromatography.

Table 1
Nucleotide content of myeloblasts from Patient W. L.
Perchloric acid extracts from myeloblasts (Patient W. L.) incubated in culture for 24 hr in the presence or absence of 9 μ M DU were analyzed by high-pressure liquid chromatography.

Nucleotide	Nucleotide concentration (nmol/10 ⁹ cells)	
	-DU	+DU
GTP	532	505
ATP	2018	1997
CTP	115	108
UTP	532	451
ADP	130	104
GDP	50	49

Table 2
Metabolites of ara-C in AML myeloblasts
Myeloblasts from AML patients were incubated for 24 hr in medium containing [5-³H]ara-C and DU as indicated. Perchloric acid extracts of the cells were analyzed by thin-layer chromatography. The cell preparation from Patient M. W. contained 93% myeloblasts, 4% lymphocytes, 1% neutrophils, and 2% others; that from Patient D. L. contained 97% myeloblasts and 3% others. Patient M. W. responded to therapy with ara-C and thioguanine.

Concentration (μ M)			Distribution of radioactivity (% of total)							
ara-C	DU	Patient	Total ara-C metabolites (nmol/10 ⁹ cells)	ara-CTP	ara-CDP	ara-CMP	ara-CDPch ^a	ara-CDPet	ara-C + ara-U	ara-U phosphates
9	8	M. W.	96.8	45.1	6.9	0.5	19.5	9.9	2.4	5.8
9	0		104.2	53.5	2.3	0.5	15.1	5.7	3.4	12.0
3	8		45.1	48.6	6.8	0.3	17.9	10.4	1.1	5.5
3	0		47.5	61.6	2.9	0.5	14.1	8.2	1.1	17.5
0.3	8		4.7	44.2	13.2	0.2	20.2	16.2	1.1	3.3
0.3	0		6.5	52.5	8.5	0.1	13.8	8.0	0.8	6.5
9	9	D. L.	168.9	45.2	6.9	1.0	20.7	5.6	8.7	6.9
9	0		258.9	54.4	5.4	0.3	19.2	6.8	5.2	5.8
3	9		107.4	44.0	7.7	1.3	27.9	7.3	2.2	5.8
3	0		260.1	55.7	7.3	0.6	12.3	8.6	2.2	10.2
0.3	9		47.2	39.2	11.5	0.6	30.2	7.3	0.8	7.4
0.3	0		52.7	59.3	19.7	0.1	8.1	3.7	0.8	4.8

^a ara-CDPch, ara-CDP-choline; ara-CDPet, ara-CDP-ethanolamine; ara-U, 1- β -D-arabinofuranosyluracil.

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presence of DU, possibly because DU-induced depletion of dCTP (1) precluded dCTP activation of dCMP deaminase (6). When myeloblasts from Patient D. L. were studied, DU decreased the anabolism of ara-C (Table 2). As a test of whether Plasmagel (used in the collection of leukemic cells) might influence the metabolism of ara-C, RPMI 6410 cells suspended in dialyzed fetal calf serum were incubated at 21° for 40 min with 0.25 volume of Plasmagel and then collected as described for cells from AML patients. Metabolism of ara-C by the RPMI 6410 cells (4) in the presence or absence of DU was not affected by the Plasmagel treatment (data not shown).

Pretreatment with DU enhanced the anabolism of ara-C approximately 3-fold in leukemia L1210 cells treated *in vivo* with [^3H]ara-C (Table 3); cells were collected 2 hr after administration of ara-C. The cellular content of ara-C metabolites in DU-treated cells was enhanced significantly (Table 3); concentrations of ara-CTP and ara-CDP-choline were increased 2- and 10-fold, respectively. In the absence of DU, ara-CTP and ara-CDP-choline accounted for 73 and 8% of acid-soluble radioactivity, respectively. In the presence of DU, the corresponding fractions were 59 and 23%, suggesting that the enhanced conversion of ara-CTP to ara-CDP-choline was a consequence of the depletion of cellular

cytidine and deoxycytidine phosphates that followed exposure to DU (1).

Liver is rich in CDP-choline (3) and phosphorylcholine cytidyltransferase (EC 2.7.7.15) (3). Table 4 lists the ara-C metabolites present in mouse liver 1.5 hr after the i.p. administration of [^3H]ara-C with or without prior treatment with DU. DU treatment did not significantly influence concentrations of ara-C metabolites in liver, with the possible exception of 1- β -D-arabinofuranosyluracil and its 5'-monophosphate, concentrations of which were slightly lower in the DU-treated mice. The summed content of ara-CDP-choline and ara-CDP-ethanolamine (Table 4, ara-CDP-X) was much greater than that of ara-CTP, suggesting a rapid conversion of the latter to the former in liver.

In LS cells, ara-CTP and ara-CDP-choline were the major anabolites of ara-C following exposure to this drug for 24 hr (Table 5). In the presence of 8 μM DU, the anabolism of 0.3 μM ara-C was enhanced 2- to 3-fold, but that of 3 μM ara-C

Table 3

Metabolites of ara-C in leukemia L1210 cells in vivo

BDF₁ mice were used 6 days after i.p. implantation of 10^5 leukemia L1210 cells; 3 mice received DU (60 mg/kg) at -3 and 0 hr and [^3H]ara-C (15 mg/kg; 20 $\mu\text{Ci}/\text{mouse}$) at 0 hr. Another group of 3 mice received 0.154 M NaCl at -3 and 0 hr and [^3H]ara-C at 0 hr. Two hr after the ara-C injection, L1210 cells from ascitic fluid were extracted with perchloric acid; the extracts were analyzed by thin-layer chromatography.

Metabolites	Cellular concentration (nmol/ 10^6 cells)	
	- DU	+ DU ^a
ara-CTP	60.6 \pm 6.7 ^b	147.0 \pm 10.2
ara-CDP	7.5 \pm 1.3	18.0 \pm 1.4
ara-CMP	2.3 \pm 0.3	5.1 \pm 0.5
ara-CDP-choline	8.5 \pm 1.3	58.1 \pm 4.8
ara-CDP-ethanolamine	2.3 \pm 0.2	14.4 \pm 3.8
ara-C	2.9 \pm 0.4	5.3 \pm 0.4
Total (unchromatographed)	83.4 \pm 9.5	250.7 \pm 17.6

^a Differs from -DU group; $p < 0.05$.

^b Mean \pm S.E. of 3 determinations.

Table 4
Metabolites of ara-C in mouse liver in vivo

Three BDF₁ mice received DU (60 mg/kg i.p.) at -3 and 0 hr and [^3H]ara-C (15 mg/kg; 20 $\mu\text{Ci}/\text{mouse}$) at 0 hr. Another group of 3 mice received 0.154 M NaCl at -3 and 0 hr and [^3H]ara-C at 0 hr. After 90 min, perchloric acid extracts were prepared from quick-frozen liver samples and analyzed by thin-layer chromatography.

Metabolite	Metabolite concentration (pmol/mg protein)	
	- DU	+ DU
ara-CTP	3.2 \pm 0.3 ^a	2.7 \pm 0.8
ara-CDP	2.4 \pm 0.3	2.0 \pm 0.5
ara-CMP	6.7 \pm 2.2	5.6 \pm 2.0
ara-CDP-X ^b	63.6 \pm 20.8	41.8 \pm 2.6
ara-C	26.4 \pm 6.3	25.3 \pm 8.8
ara-UTP	4.4 \pm 1.3	3.4 \pm 0.5
ara-UDP	4.1 \pm 0.7	2.5 \pm 0.8
ara-UMP	23.1 \pm 4.9	11.6 \pm 1.2 ^c
ara-U	65.8 \pm 16.3	33.2 \pm 3.4 ^c
Total (unchromatographed)	173.6 \pm 32.4	112.9 \pm 14.1

^a Mean \pm S.E. of 3 determinations.

^b ara-CDP-X, ara-CDP-choline and ara-CDP-ethanolamine; ara-UTP, 1- β -D-arabinofuranosyluracil triphosphate; ara-UDP, 1- β -D-arabinofuranosyluracil diphosphate; ara-UMP, 1- β -D-arabinofuranosyluracil monophosphate; ara-U, 1- β -D-arabinofuranosyluracil.

^c Differs from -DU group; $p < 0.10$.

Table 5

Metabolites of ara-C in LS lymphocytes

Cells were cultured for 24 hr in the presence of the indicated concentrations of [^3H]ara-C and DU. Perchloric acid extracts of the cells were analyzed by thin-layer chromatography.

Concentration (μM)		Total ara-C metabolites (nmol/ 10^6 cells)		Distribution of radioactivity (% of total) in Experiment 2					
ara-C	DU	Experiment 1	Experiment 2	ara-CTP	ara-CDP	ara-CMP	ara-CDPch ^a	ara-CDPet	ara-C + ara-U
3	0	17.21	30.94	39	18	2	25	4	6
3	8	20.93	38.61	38	15	2	30	5	5
0.3	0	3.15	5.24	40	18	2	28	3	3
0.3	8	6.44	14.43	37	16	2	34	4	3

^a ara-CDPch, ara-CDP-choline; ara-CDPet, ara-CDP-ethanolamine; ara-U, 1- β -D-arabinofuranosyluracil.

ara-CDP-Choline in Neoplastic and Normal Cells

Table 6

Metabolites of ara-C in murine L5178Y cells

L5178Y cells were cultured in [5-³H]ara-C and DU as indicated for 20 hr. Perchloric acid extracts of the cells were analyzed by thin-layer chromatography.

Concentration (μM)		Total ara-C metabolites (nmol/10 ⁶ cells)	Distribution of radioactivity (% of total)						
ara-C	DU		ara-CTP	ara-CDP	ara-CMP	ara-CDPch ^a	ara-CDPet	ara-C + ara-U	ara-U phosphates
0.4	0	11.06	80.8	5.0	0.7	2.1	0	1.9	3.4
0.4	2.7	12.93	75.5	3.4	0.7	8.6	0.4	3.9	3.8
2.7	0	36.36	80.5	4.3	1.1	3.0	0	3.8	1.6
2.7	2.7	22.17	76.9	1.3	0	6.2	1.2	3.3	0.7
10	0	58.75	78.1	3.9	1.3	2.4	1.2	5.7	4.7
10	2.7	51.09	75.2	7.6	1.5	7.5	0	3.9	6.4

^a ara-CDPch, ara-CDP-choline; ara-CDPet, ara-CDP-ethanolamine; ara-U, 1-β-D-arabinofuranosyluracil.

Table 7

Metabolites of ara-C in HeLa cells

HeLa cells were cultured in the presence of [5-³H]ara-C and DU as indicated for 24 hr. Perchloric acid extracts of the cells were analyzed by thin-layer chromatography.

Concentration (μM)		Total ara-C metabolites (nmol/10 ⁶ cells)	Distribution of radioactivity (% of total)						
ara-C	DU		ara-CTP	ara-CDP	ara-CMP	ara-CDPch ^a	ara-CDPet	ara-C + ara-U	
0.1	0	0.50	66.9	3.3	2.0	17.0	1.4	4.3	
0.1	10	0.50	51.8	5.8	5.0	20.4	3.0	5.9	
1	0	2.29	55.7	7.1	4.3	13.7	0.9	10.0	
1	10	1.96	54.5	6.1	4.1	17.9	3.6	6.8	

^a ara-CDPch, ara-CDP-choline; ara-CDPet, ara-CDP-ethanolamine; ara-U, 1-β-D-arabinofuranosyluracil.

was enhanced only marginally. ara-CDP-choline and ara-CDP-ethanolamine were metabolites of ara-C in LS cells, but in contrast to RPMI 6410 cells the proportion of ara-C radioactivity associated with ara-CDP-choline increased only modestly in the presence of DU.

When cells of the murine lymphoma L5178Y were cultured for 20 hr in medium containing [5-³H]ara-C in the presence or absence of DU, ara-CDP-choline was a minor metabolite of ara-C (Table 6). Synthesis of this metabolite was increased 2- to 4-fold when DU was present; however, DU did not influence the total anabolism of ara-C under the conditions described in Table 6. Likewise, DU did not affect the anabolism of ara-C in HeLa cells (Table 7); however, ara-CDP-choline was a substantial metabolite of ara-C in these cells (Table 7). Under certain conditions, the combination of ara-C and DU can be more toxic toward HeLa cells than can the sum of the effects of the individual agents (7).

The present results show that ara-CDP-choline is a significant metabolite of ara-C in several cell types in the presence or absence of DU. Further exploration will be required to evaluate the possible contribution of this metabolite to ara-C toxicity.

The effects of DU on ara-C anabolism were not consistent in the different types of cells used here; the reason for this is not known. A better understanding of the biochemical mechanisms underlying the DU enhancement of ara-C ana-

bolism may help to explain this apparent selectivity of DU effects. We suggest that a DU-induced increase of ara-C anabolism is central to the synergistic enhancement of toxicity of DU/ara-C combinations (4).

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